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Respuestas asociadas al déficit hídrico en leguminosas: acumulación de prolina y estrés nitro-oxidativo

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INDICE

RESUMEN

La sequía afecta el crecimiento y productividad de las plantas, y a nivel celular induce ajustes metabólicos y sobreproducción de especies reactivas del oxígeno (ROS). Entre los cambios metabólicos la acumulación de prolina (Pro) es una respuesta conservada en distintas especies de plantas frente a diferentes estreses. Hasta el momento no es claro el rol de la Pro durante el estrés, aunque durante mucho tiempo se la ha considerado un osmolito compatible con actividad antioxidante.

En el capítulo 1 de esta tesis se compararon dos leguminosas forrajeras con tolerancia estival contrastante, trébol y lotus. Estas leguminosas respondieron de manera diferente en el control de la pérdida de agua, acumulación de prolina y actividad antioxidante enzimática y del fotosistema II. En lotus en condición de sequía, se indujeron las enzimas Mn-SOD y Fe-SOD y en trébol la actividad SOD total fue inferior a la de lotus. A su vez, la combinación de sequía y calor en lotus produjo descenso en la actividad del fotosistema II y degradación de la proteína D2. Esto permite suponer que el descenso de la actividad fotosintética, sin entrar en marchites permanente, puede ser una ventaja de lotus en la disipación de la energía lumínica y en la economía del agua.

En la leguminosa modelo *Lotus japonicus* se evaluó el estrés nitro-oxidativo en condición de sequía. Los resultados evidenciaron que en hojas el estrés oxidativo es mayor que en raíces, mientras que en éstas es mayor el estrés nitrosativo, donde determinamos un patrón de proteínas nitradas específico. También se identificaron dos genes *p5cs*, que participan en la síntesis de Pro, que definimos como *Ljp5cs1*, inducible por estrés osmótico, y *Ljp5cs2* constitutivo.

En el capítulo 2 se analizó la capacidad antioxidante de la Pro. Mediante estudios *in sillico* determinamos que las barreras de reacción entre la Pro y el radical hidroxilo (·OH) son entre 3 y 9 kcal/mol. A su vez propusimos un ciclo Pro-Pro, en el cual la Pro capta ·OH y rinde Pro radical, que reacciona con un segundo ·OH y forma P5C que por catálisis enzimática con consumo de NADPH regenera Pro. Por otro lado también demostramos que si el ·OH abstrae un H del grupo NH_2^+ de la Pro, se produce la decarboxilación de la misma, generando un precursor del ácido γ-amino butírico (GABA), compuesto que también se acumula en condiciones de estrés.

Mediante estudios *in vitro*, demostramos que la Pro no es capaz de extinguir oxígeno singulete como se consideró durante muchos años. A su vez aportamos evidencias de que la Pro es incapaz de atenuar la nitración de proteínas mediada por peroxinitrito, superóxido, óxido nítrico y dióxido de nitrógeno. Estos resultados llevan a reconsiderar el rol antioxidante que se le ha asignado a la Pro durante varios años, y contribuyen a orientar el planteo de hipótesis en cuanto a su funcionalidad.

INTRODUCCIÓN

La familia Leguminosae incluye especies de importancia agronómica

La principal fuente de proteína en la dieta humana es de origen animal y la producción ganadera se basa en pasturas naturales o granos (*feedlot*). Entre las pasturas, varias especies de leguminosas son usadas en todo el mundo como forraje debido a su capacidad de establecer asociaciones simbióticas con bacterias capaces de fijar nitrógeno y por su alto contenido de proteínas y minerales (Ca^{+2} y Mg^{+2}). Por esto los cultivos de leguminosas juegan un rol importante como fuente de proteínas, principalmente en regiones con suelos de baja fertilidad.

Para aumentar la productividad de las leguminosas, un aspecto a considerar es la mejora de la tolerancia a estreses abióticos. La identificación de características fisiológicas y bioquímicas capaces de aumentar la producción de leguminosas en condiciones limitantes de crecimiento es el principal objetivo de los mejoradores de plantas para la agricultura y la ganadería. En esta tesis se propone explicar algunos mecanismos de tolerancia a estrés abiótico en leguminosas.

Lotus japonicus es una leguminosa modelo

Lotus japonicus es utilizada como leguminosa modelo, por tener un genoma simple, ciclo de vida corto y contar con la secuenciación de su genoma completo (Sandal et al., 2002; Sato et al., 2001). A su vez, una serie de recursos y herramientas genéticas como ecotipos, líneas de mutantes, mapas genéticos, RIL (*recombinant inbred lines*) y EST (*expressed sequence tags*) se han hecho disponibles (VandenBosch and Stacey, 2003).

Esta leguminosa modelo está emparentada con las leguminosas forrajeras *Lotus corniculatus*, *Lotus uliginosus, Lotus glaber y Lotus subbiflorus* (Choi et al., 2004; Díaz et al., 2005), ampliamente utilizadas en la producción animal. Por lo expuesto *L. japonicus* es un modelo útil para comprender y mejorar especies de uso agronómico.

El estrés abiótico genera diferentes respuestas en las plantas

Las plantas se encuentran sometidas a condiciones ambientales que generan diferentes tipos de estrés, que afectan su crecimiento, desarrollo y productividad (Boyer, 1982). Entre las condiciones ambientales que pueden generar estrés abiótico se encuentra el exceso o déficit de agua, así como niveles inadecuados de salinidad, temperatura, concentración de minerales e irradiación (Bray et al., 2000).

El déficit hídrico, o sequía, se establece cuando la tasa de transpiración excede a la de absorción de agua. La habilidad para tolerar la sequía está determinada por múltiples genes de vías bioquímicas responsables de productos o procesos que mejoran la tolerancia actuando aditivamente y a veces sinérgicamente (Bohnert et al., 1995). En general, y a diferencia del estrés biótico, la tolerancia a estreses abióticos no es el resultado de la acción de un gen específico (Zhu et al., 1997).

La sequía induce cambios morfológicos, fisiológicos y bioquímicos (Lawlor and Cornic, 2002), que tienen como consecuencia la limitación del crecimiento y desarrollo de las plantas lo que puede llevar a significantes pérdidas en la productividad y calidad de granos, así como de la producción ganadera y lechera (McClaran and Wei, 2014). A nivel celular la sequía puede resultar en el incremento de la concentración de solutos, cambios en el volumen celular y alteración del potencial de agua, entre otros (Bray, 1997). Así, la habilidad de las plantas para responder y sobrevivir a la sequía depende de mecanismos que involucran la integración de respuestas celulares a través de toda la planta (Bray et al., 2000).

Las plantas sobreproducen ROS/RNS en condición de sequía

La aclimatación de las plantas a la sequía está asociada con elevados niveles de especies reactivas del oxígeno (ROS), como el peróxido de hidrógeno (H_2O_2), radical superóxido ($O_2^{\cdot-}$), oxigeno singulete ($^1O_2$) y radical hidroxilo ($^{\cdot}OH$) los cuales son responsables del daño oxidativo asociado al estrés (Dat et al., 2000; Smirnoff, 1993).

En las plantas, al igual que en otros organismos, en las mitocondrias y peroxisomas se producen altos niveles de ROS. A su vez, en los cloroplastos también se generan

ROS, como consecuencia de la alta concentración de oxígeno producido por la actividad fotosintética y de la excitación de electrones de pigmentos por la luz (Fig. 1).

En condición de déficit hídrico y/o altas temperaturas, la actividad del ciclo de Calvin disminuye, lo que previene la oxidación de NADPH y regeneración de $NADP^+$. Cuando se combina la falta de $NADP^+$ con alta intensidad lumínica, los electrones de las clorofilas producen $^1O_2$ en los centros de reacción y el flujo de electrones acumulación de ROS (Chaves et al., 2009).

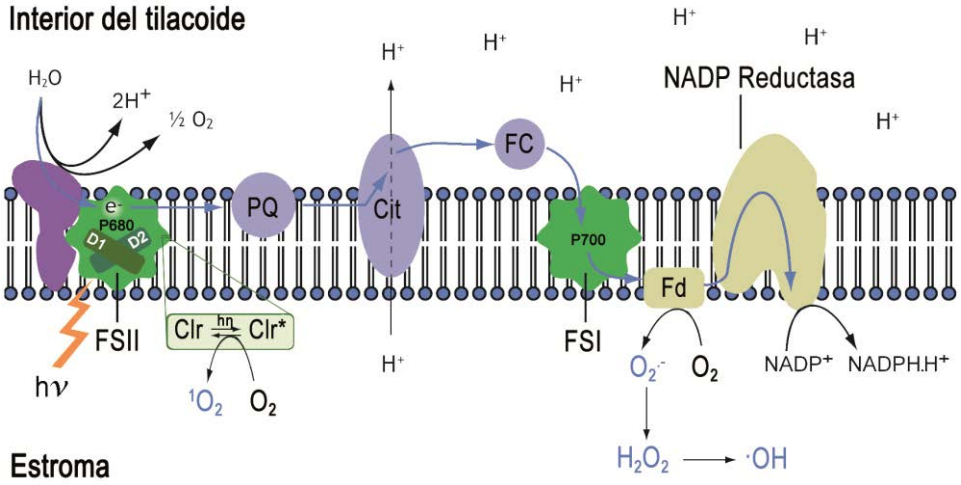

Figura 1. Producción de ROS en cloroplastos. Clr, clorofila; Clr*, clorofila excitada; FC, ficocianina; Fd, ferredoxina; FSI, fotosistema I; FSII, fotosistema II; Cit, citocromo, PQ, plastoquinona. El superóxido ($O_2^{\cdot -}$) puede ser producido a partir del O_2 por transferencia electrónica desde la Fd reducida, un potente agente reductor. El peróxido de hidrógeno (H_2O_2) es producido por dismutación del $O_2^{\cdot -}$ ya sea espontánea o catalizada por la SOD cloroplástica. El radical hidroxilo ($^{\cdot}OH$) se genera por homólisis del H_2O_2 en presencia de luz UV-Vis, o por reacción de Fenton en presencia de Fe^{3+}. El oxígeno singulete ($^1O_2$) es principalmente generado por transferencia de energía de clorofilas excitadas.

Por otra parte, las especies reactivas del nitrógeno (RNS) como el óxido nítrico ($^{\cdot}NO$) y sus derivados, peroxinitrito ($ONOO^-$) y dióxido de nitrógeno ($^{\cdot}NO_2$), han tomado importancia en los últimos años en la biología de plantas. Se han descrito al menos siete vías biosintéticas de $^{\cdot}NO$ en plantas, pero sólo se ha logrado caracterizar los componentes moleculares y enzimáticos de una de ellas (Gupta et al., 2011).

El $^{\cdot}NO$ y el $O_2^{\cdot-}$ se combinan rápidamente en una reacción controlada por difusión para generar $ONOO^-$. Esta molécula es un potente oxidante y su ácido, el ácido peroxinitroso (ONOOH), produce espontáneamente $^{\cdot}OH$ y $^{\cdot}NO_2$ por homólisis (Beckman et al., 1990). Varios trabajos han demostrado que las RNS también se incrementan en diversas situaciones de estrés (Begara-Morales et al., 2013; Chaki et al., 2013; Corpas and Barroso, 2013).

La sequía promueve respuestas adaptativas como la foto-inhibición

El sistema fotosintético está diseñado para absorber grandes cantidades de energía lumínica y convertirla en energía química. La energía lumínica en exceso, puede aumentar la producción de ROS que producen daño, si no se disipa de manera segura (Asada, 1999; Horton et al., 1996; Müller et al., 2001). Para esto las plantas cuentan con mecanismos de reparación y regulación complejos (Fig. 2). En la primera línea de defensa, los carotenoides juegan un rol clave al capturar (*quenching*) rápidamente el estado excitado de las clorofilas, lo que evita la formación de $^1O_2$. Los carotenoides no tienen la energía suficiente para formar oxígeno singulete por lo que decaen al estado basal perdiendo la energía como calor (Fig. 2).

Otra forma de protección de la estabilidad del aparato fotosintético es la foto-inhibición, definida como la inhibición de la fotosíntesis por exceso de luz (Taiz and Zeiger, 2002). La foto-inhibición es reversible en estadios tempranos, sin embargo la inhibición prolongada resulta en el desensamblaje y reparación del centro de reacción (Melis, 1999). El principal blanco de este daño es la proteína D1 que cubre parte del centro de reacción del FSII (Fig. 1). Cuando la D1 es dañada, esta debe ser removida de la membrana y remplazada por una molécula sintetizada *de novo* (Fig. 2). Se cree que las otras piezas del fotosistema son recicladas y que solo la D1 se sintetiza de nuevo (Hu et al., 2004; Ohad et al., 1984). El daño foto-inhibitorio observado es el resultado neto del balance entre el foto-daño y la reparación (Lidholm et al., 1987; Samuelsson et al., 1985).

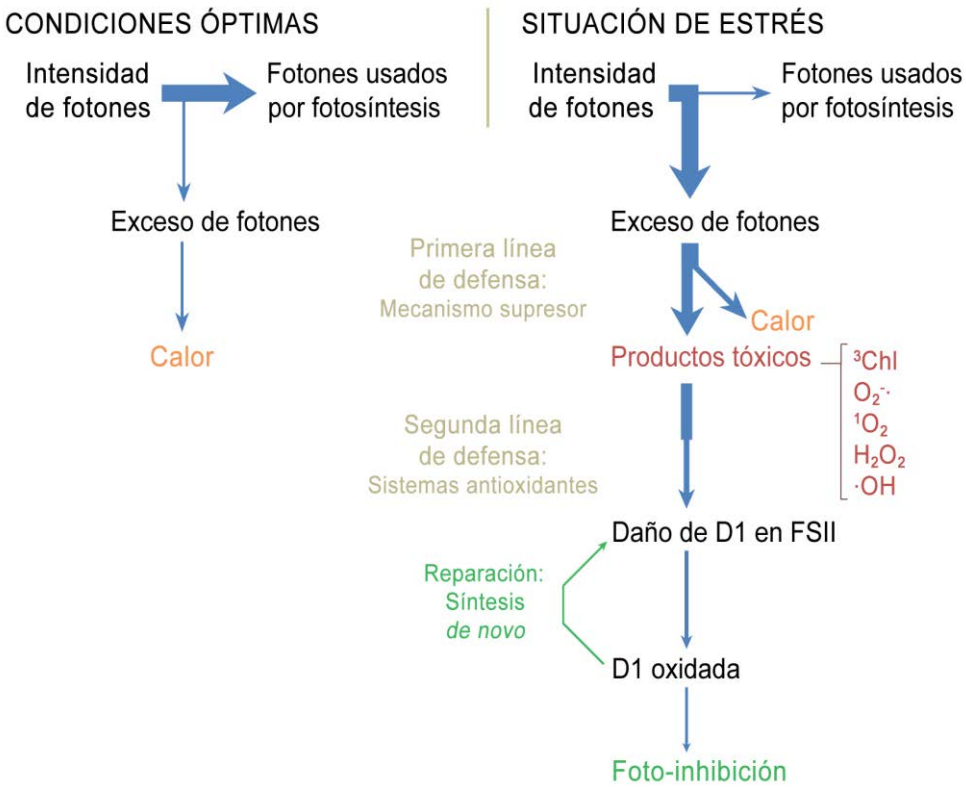

Figura 2. Esquema del mecanismo de protección y reparación del fotosistema II. La protección del foto-daño implica dos líneas de defensa. La primera línea corresponde a la captura del exceso de excitación como calor. En situaciones de estrés esta defensa no es suficiente y se forman productos tóxicos. La segunda línea de defensa elimina estos productos tóxicos mediante diferentes sistemas antioxidantes. Si esta línea de defensa es insuficiente, se daña la proteína D1 del FSII y se produce la foto-inhibición. La D1 es removida del centro de reacción del FSII y es degradada. Una nueva D1 es sintetizada y reinsertada en el centro de reacción del FSII para formar una unidad funcional.

La sequía favorece la foto-inhibición y la combinación con otro estrés abiótico, como la alta temperatura, potencia este proceso (Takahashi and Murata, 2008). Varios estudios han evidenciado cambios en los parámetros de fluorescencia de clorofilas en respuesta a estreses ambientales como el calor, el frío, la helada y la salinidad (Smillie and Hetherington, 1983). Otros estudios han demostrado una correlación entre el decrecimiento en el rendimiento cuántico máximo del FSII (F_V/F_M) y la disociación física del centro de reacción del FSII producido por foto-inhibición, y utilizan esta técnica para discriminar cultivares tolerantes (Bonnecarrere et al., 2011).

Los sistemas antioxidantes protegen a las plantas del daño oxidativo

Las plantas controlan los niveles de ROS mediante diferentes sistemas antioxidantes enzimáticos y no enzimáticos. En respuesta enzimática al estrés oxidativo se pueden encontrar inducidas las distintas isoformas superóxido dismutasa (SOD), la catalasa (CAT), las actividades del ciclo ascorbato-glutatión como la glutatión reductasa (GR) o la ascorbato peroxidasa (APX), quienes confieren mayor tolerancia frente a estreses ambientales específicos (Sade et al., 2011). En la figura 3 se esquematiza la acción de las principales enzimas antioxidantes y su localización sub-celular.

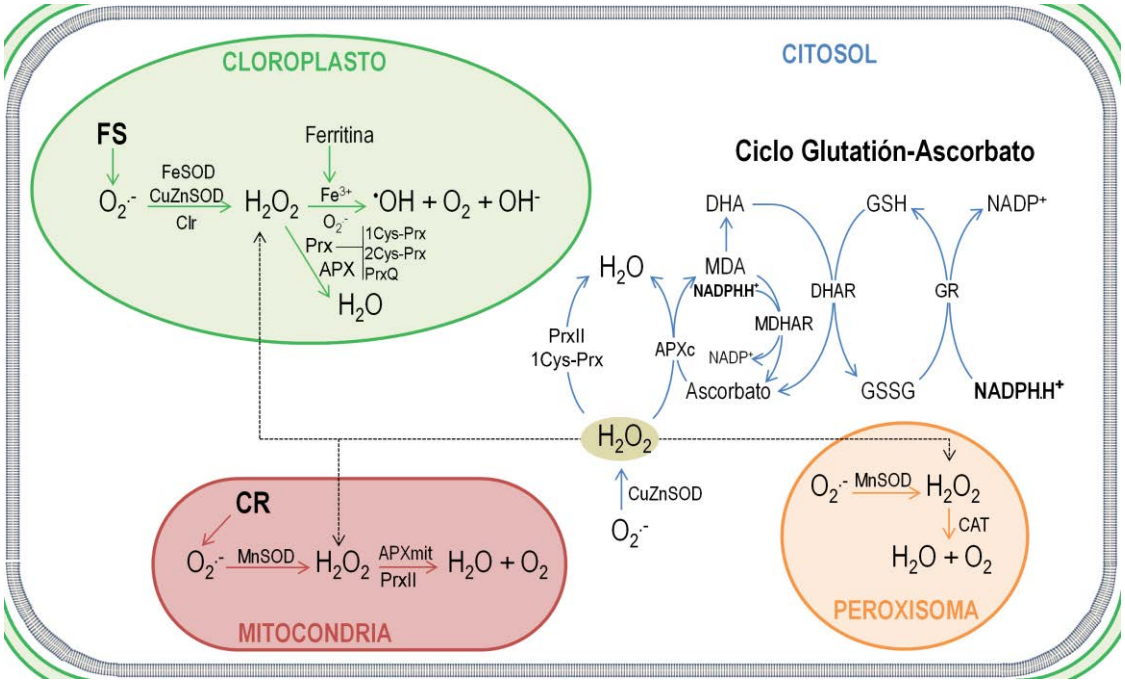

Figura 3. Principales enzimas antioxidantes en plantas. CAT, catalasa; SOD, superóxido dismutasa; APX, ascorbato peroxidasa, GR, glutatión reductasa; GSH, glutatión reducido; GSSG, glutatión oxidado; Prx, perredoxinas; MDA, monodehidroascorbato; MDHAR, monodehidroascorbato reductasa; DHAR, dehidroascorbato reductasa; DHA, dehidroascorbato. Clr, clorofila; CR, cadena respiratoria; FS, fotosistema.

Las actividades enzimáticas antioxidantes son analizadas comúnmente en varias especies ya que pueden ser utilizadas como marcadores de tolerancia a estrés, ya que su inducción está asociada a la aclimatación al estrés. Así, en cultivares tolerantes a sequía de trigo (Al-ghamdi, 2009), tomate (Sánchez-Rodríguez et al., 2012), alfalfa (Wang et al., 2009) y manzana (Wang et al., 2012) se ha observado una mayor

inducción de estas enzimas cuando se las comparó con un cultivar sensible.

Entre los antioxidantes solubles no enzimáticos se incluye el glutatión (GSH), ascorbato, carotenos y tocoferol, que en respuesta al estrés oxidativo inducido por sequía se encuentran en niveles elevados (Feng et al., 2004). Estas moléculas son preferentemente oxidadas por especies reactivas respecto a otras biomoléculas como lípidos de membrana, proteínas, entre otros. El glutatión puede ser transformado en nitrosoglutation (GSNO) por nitrosilación de la cisteína mediada por $^{\cdot}NO$. Dado que la nitrosilación es un proceso reversible, el nitrosoglutation es considerado un reservorio móvil de $^{\cdot}NO$ en plantas (Barroso et al., 2006). La enzima nitrosoglutatión reductasa (GSNOR) degrada el nitrosoglutatión (GSNO) en glutatión oxidado (GSSG) y NH_3 (Airaki et al., 2011). También el ascorbato puede capturar varias especies reactivas como el $O_2^{\cdot-}$, $^{\cdot}OH$, $RO_2^{\cdot}$ y $ONOO^-$ (Halliwell, 2006).

Durante muchos años se ha considerado a la prolina entre los antioxidantes no enzimáticos de bajo peso molecular (Alia et al., 1991; Chen and Dickman, 2005; Kavi Kishor and Sreenivasulu, 2014; Rejeb et al., 2014; Smirnoff and Cumbes, 1989; Szabados and Savouré, 2010). Esta función es re-evaluada y discutida a lo largo de esta tesis.

Otro aspecto a considerar en la defensa antioxidante es el abastecimiento de los equivalentes de reducción en forma de NADPH (Mateos et al., 2009). Como se observa en la figura 3 el NADPH es requerido por 2 enzimas del ciclo glutatión-ascorbato, que mantiene el ascorbato y GSH necesario para la protección contra el daño oxidativo en plantas (Corpas et al., 1998; Noctor and Foyer, 1998; Noctor, 2006).

El NADPH también es usado por la tiorredoxina reductasa en la regulación de vías metabólicas mediante la reducción de un grupo tiol (Nordman et al., 2003) y en la formación de tetrámeros de catalasa activa, donde cada monómero de enzima contiene ligado un NADPH (Kirkman and Gaetani, 2007). Por ello, la capacidad antioxidante de la célula puede estar limitada por la disponibilidad de NADPH. Las principales enzimas encargadas de generar poder reductor en forma de NADPH en

células de plantas son, a nivel citoplasmático la glucosa-6-fosfato deshidrogenasa (G6PDH) y la 6-fosfogluconato deshidrogenasa (6PGDH), ambas implicadas en la vía de las pentosas fosfato, la NADP-málica (NADP-ME) y la NADP-isocitrato deshidrogenasa (NADP-ICDH) y a nivel cloroplástico la ferredoxina-NADP reductasa (Fd-NADP) (Noctor et al., 2006; Valderrama et al., 2006).

El exceso de ROS/RNS produce estrés nitro-oxidativo

En un principio las ROS se consideraron productos tóxicos del metabolismo aeróbico, sin embargo se ha demostrado que tanto las ROS como el ·NO tienen una función en la respuesta de defensa frente a patógenos y en la señalización para producir la aclimatación a diversos estreses abióticos (Dat et al., 2000; Mittler et al., 2011; Molassiotis et al., 2010). Por ejemplo dentro de las ROS, el H_2O_2 que puede difundir a través de las membranas (Fig. 3) funciona como una de las principales moléculas señal en plantas (Miller et al., 2008). Se cree que la expresión de al menos 2% de los genes de Arabidopsis (algunos de ellos enzimas antioxidantes claves) es dependiente de H_2O_2 (Desikan et al., 2001). Entre las RNS, el ·NO se ha relacionado con procesos de señalización, inhibición de la germinación, movimiento estomático, senescencia, respuesta a estrés abiótico, entre otros (León et al., 2014). El rol del ·NO en el estrés abiótico como señalizador se ha destacado, ya que se demostró que al igual que el H_2O_2, está involucrado en la adaptación dado que pre-exposiciones a estas moléculas pueden inducir la tolerancia frente a sucesivos estreses más severos (Jasid et al., 2006; Tanou et al., 2009). Por ello, las especies reactivas tienen un efecto positivo cuando sus concentraciones son bajas, cuando los sistemas de defensa antioxidante proveen una adecuada protección. Sin embargo, cuando la producción de ROS y RNS supera la capacidad de los sistemas antioxidantes se establece estrés nitro-oxidativo. El daño celular causado en esta situación incluye la oxidación o nitración de lípidos de membrana, proteínas y ácidos nucleicos. Gran parte de este tipo de daño depende de la compartimentación por membranas, que ofrecen una significativa resistencia al pasaje de la mayoría de estas especies reactivas (Möller et al., 2008). Sin embargo el $^1O_2$, el ·NO (Denicola et al., 1996b) y el $\cdot NO_2$ (Signorelli et al., 2011) pueden difundir a

través de las membranas y reaccionar con antioxidantes de bajo peso molecular, lípidos insaturados y proteínas de diferentes compartimentos.

Si bien nos referimos a estrés nitro-oxidativo cuando se establece una situación de estrés oxidativo y nitrosativo, estos estreses se determinan experimentalmente utilizando distintos marcadores. La peroxidación lipídica es utilizada comúnmente como marcador de estrés oxidativo, y en sequía se ha observado que la misma incrementa en varios tejidos (Sade et al., 2011). El marcador de estrés nitrosativo más usado es la nitración de residuos de tirosinas de proteínas (Corpas et al., 2007; Radi, 2004).

Se ha propuesto que la nitración de residuos de tirosinas puede causar cambios en la función de las proteínas, ya sea produciendo la pérdida de función, como ocurre con la MnSOD y la GR (Souza et al., 2008), o la ganancia de función como ocurre con la activación de la actividad peroxidatica del citocromo c (Batthyany et al., 2005).

En general, la nitración de enzimas en plantas conduce su inactivación (Corpas and Barroso, 2013). A altas temperaturas la Fd-NADP reductasa y la anhidrasa carbónica, enzimas que participan en la asimilación del carbono, se inactivan por nitración de tirosinas (Chaki et al., 2013, 2011b). También se ha observado que la nitración de la NADP-ICDH de arveja pierde actividad al ser nitrada, un proceso que ocurre comúnmente en la senescencia (Begara-Morales et al., 2014, 2013).

Otras modificaciones mediadas por las RNS son la formación de nitro-alcanos y la *S*-nitrosilación de proteínas, esta última es de particular interés debido a que puede estar involucrada en la regulación reversible de la función de proteínas, así como contribuir al daño por estrés nitrosativo (Cornwell et al., 2003; Marshall and Stamler, 2001).

Recientemente se ha demostrado que las RNS pueden tener un doble rol sobre la actividad de las enzimas, ya que si bien la APX de arveja puede ser inactivada irreversiblemente por nitración, su actividad es potenciada reversiblemente por nitrosilación (Begara-Morales et al., 2014).

Las leguminosas acumulan prolina en respuesta a sequía

En respuesta a la sequía las células de las plantas acumulan en el citoplasma compuestos de bajo peso molecular denominados solutos compatibles, como prolina, glicín betaina, azúcares y polioles, para controlar el balance iónico en la vacuola (Parida and Das, 2005). La acumulación de prolina también se ha reportado en condiciones alta salinidad, exposición a alta intensidad lumínica, metales pesados, estrés oxidativo y diversos estreses abióticos (Szabados and Savouré, 2010).

En plantas superiores la prolina es sintetizada principalmente a partir del glutamato que es reducido a glutamato-semialdehido (GSA) por la enzima pirrolin-5-carboxilato sintetasa (P5CS). Este se convierte espontáneamente en pirrolin-5-carboxilato (P5C) (Hu et al., 1992; Savoure et al., 1995), figura 4. Por último la enzima P5C reductasa (P5CR) reduce el P5C a prolina. Esta vía ocurre en el citosol y en el estroma del cloroplasto con consumo de NAD(P)H.H$^+$. Como vía alternativa la prolina también puede ser sintetizada a partir de ornitina, la cual es transformada en GSA por acción de la ornitín-aminotransferasa (OAT), que espontáneamente dará P5C y luego éste prolina (Delauney and Verma, 1993; Roosens et al., 1998).

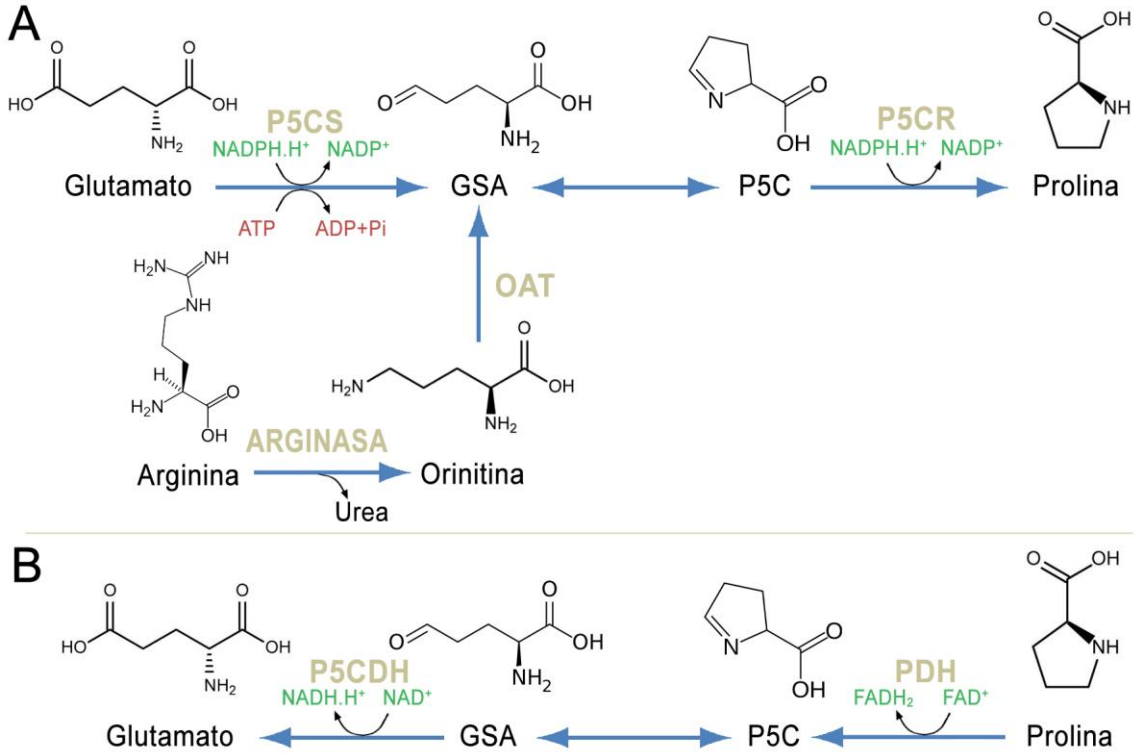

Figura 4. Metabolismo de prolina. A. Síntesis de prolina. P5CS, pirrolin-5-carboxilato sintetasa; P5CR, pirrolin-5-carboxilato reductasa; OAT, ornitín amino transferasa. B. Degradación de prolina. P5CDH, pirrolin-5-carboxilato deshidrogenasa; PDH, prolina deshidrogenasa. GSA, glutamato-semialdehido; P5C, pirrolin 5-carboxilato.

En la mayoría de las especies la enzima P5CS está codificada por 2 genes, mientras que la P5CR está codificada por uno (Szabados and Savouré, 2010). La acumulación de prolina en plantas es controlada por la acción de los genes P5CS, donde uno actúa como *housekeeping* y el otro es una isoforma especifica de estrés. Estos genes a pesar de ser muy similares en su secuencia la regulación transcripcional es diferente (Armengaud et al., 2004; Strizhov et al., 1997).

Con el fin de contar con una herramienta para evaluar el rol de la prolina en estrés hídrico, en el laboratorio de Bioquímica de Facultad de Agronomía se desarrolló un transgénico de *L. japonicus* con silenciamiento de un gen *p5cs* mediante RNAi. En el Capítulo 1 de esta tesis se presentarán los resultados obtenidos al evaluar el transgénico y las conclusiones sobre los genes que participan en la síntesis de prolina en *L. japonicus*.

Se ha demostrado que la mayor concentración de prolina se logra en cloroplastos, alcanzando concentraciones de 160 mM, incluso en situaciones no estresantes (Büssis and Heineke, 1998). Una explicación es que la proteína P5CS inducible por estrés se movilice hacia este organelo en dicha condición. Fusiones de *green fluorescent protein* (GFP) con la proteína P5CS1 de *A. thaliana* demostraron que en situación de estrés esta proteína se acumula en el cloroplasto (Szekely et al., 2008).

La prolina: un aminoácido con múltiples funciones en plantas

La capacidad de acumular y utilizar prolina por algunos genotipos se ha relacionado con mayor tolerancia a la sequía (Al-Sulaiti et al., 1990; Hare et al., 1998), aunque no hay evidencias sólidas de que plantas que acumulan más prolina sean más tolerantes al déficit hídrico (Choudhary et al., 2005; Samaras et al., 1995).

Si bien la acumulación de prolina en condiciones de estrés se conoce desde hace mucho tiempo (Barnnet and Naylor, 1966) y se han descrito numerosas especies

vegetales que la acumulan en respuesta a diferentes estreses (Chiang and Dandekar, 1995; Delauney and Verma, 1993; Samaras et al., 1995), no se le ha podido asignar a la prolina una función en la respuesta a estrés. Por esto, se han manejado diversas hipótesis sobre funciones protectoras de la prolina, como: captador de especies reactivas, amortiguador celular del estatus redox y del pH celular, y estabilizador de estructuras subcelulares especialmente durante el estrés osmótico y salino (Szabados and Savouré, 2010).

La prolina fue considerada por mucho tiempo un osmolito compatible, capaz de proteger estructuras subcelulares y macromoléculas del estrés osmótico (Csonka et al., 1988; Hare et al., 1997; Kavi Kishor and Sreenivasulu, 2014). A su vez, se ha demostrado que la prolina funciona como una chaperona molecular capaz de proteger la integridad de proteínas e incrementar la actividad de diferentes enzimas (Szabados and Savouré, 2010).

La mayoría de los estudios en plantas en condiciones de estrés le atribuyen a la prolina funciones antioxidantes. Esta hipótesis se extiende a levaduras y otros hongos, en los que aplicaciones exógenas de prolina previenen la muerte celular programada (Chen and Dickman, 2005), y en células humanas en las que protegería frente a estrés oxidativo, potencialmente carcinogénico (Krishnan et al., 2008). En el Capítulo 2 se hará referencia en particular a las evidencias y avances referidos al rol antioxidante de la prolina en plantas.

Otros autores proponen que la clave de la acumulación de prolina estaría en su anabolismo – catabolismo, que permitirían mantener un balance redox adecuado para la planta (Sharma et al., 2011). Estos autores sugieren que la prolina se sintetiza en los tejidos fotosintéticos para regenerar $NADP^+$, necesario para que ocurra la fotosíntesis, mientras que su catabolismo sería necesario en las células en expansión y meristemáticas para mantener el desarrollo de la planta mediante el aporte de $NADH.H^+$ y $FADH_2$ a la cadena respiratoria (Hare et al., 1997; Kavi Kishor et al., 2005; Sharma et al., 2011).

También se ha vinculado a la prolina con funciones regulatorias, como el control del desarrollo de las plantas y como molécula señal. En particular se cree que la prolina regula la división celular y la embriogénesis (Mattioli et al., 2009). Se ha observado que elevados niveles de prolina pueden llevar a un floración temprana y bajos niveles a retrasos o dificultades de floración (Mattioli et al., 2009, 2008).

Defensa antioxidante mediada por prolina

Una hipótesis manejada en los últimos 25 años es que la prolina actúa como captador de especies radicalarias, y hay evidencias de que es un eficiente captador de radical $^{\cdot}OH$ (Smirnoff & Cumbes, 1989). Sin embargo, no se conocía el mecanismo por el cual la prolina podría capturar $^{\cdot}OH$, ni los posibles productos de su reacción. También desde hace años se ha propuesto que la prolina es un eficiente extintor del $^1O_2$ (Alia et al., 2001, 1991; Matysik et al., 2002) pero los estudios no establecieron si la prolina actuaba por *quenching* físico o químico. En esta tesis, aprovechando los avances metodológicos, se realizaron estudios con una metodología más adecuada con el fin de responder cómo la prolina protege frente al $^1O_2$.

Por otra parte, han surgido evidencias que permitieron plantear que plantas transgénicas que acumulan más prolina presentan menor daño oxidativo bajo estrés hídrico (Hong et al., 2000; Hoque et al., 2006). También se demostró que plantas mutantes en *p5cs1* que acumulan menos prolina, tienen mayor acumulación de ROS y daño oxidativo (Szekely et al., 2008). Sin embargo estas evidencias son indirectas, e insuficientes para concluir que la protección de la prolina se debe a su capacidad de reaccionar con ROS/RNS. De hecho, en algunos trabajos se sugiere que la prolina protege a enzimas antioxidantes y mantiene un elevado estatus de glutatión reducido (Hoque et al., 2008, 2007; Islam et al., 2009). A su vez, la acumulación de prolina puede contribuir con la defensa antioxidante porque en su síntesis se regenera $NADP^+$ necesario como aceptor de electrones, lo que mantiene el flujo electrónico fotosintético disminuyendo la foto-inhibición y el daño del aparato fotosintético (Hare et al., 1997).

En oposición, otros autores proponen que la acumulación de prolina en plantas sometidas a alta temperatura puede resultar en mayor producción de ROS (Lv et al., 2011). Esta evidencia surge de pre-tratar con prolina exógena (50 mM) plantas transgénicas que sobre-expresan el gen *p5cs* en situación de estrés térmico y observar que en dicha condición produjeron más ROS. Estos autores consideran que el exceso de prolina genera la producción de ROS vía el ciclo Pro/P5C. Este ciclo es conocido en células de mamíferos y fue reportado en células de plantas (Miller et al., 2009). En la figura 5 se representa el ciclo Pro/P5C, que ayuda a mantener un equilibrio de pro-p5c que puede cumplir roles en algún proceso fisiológico (Miller et al., 2009). Cuando la prolina es sobre-producida la actividad del ciclo podría incrementar y esto aumentar la posibilidad de que un electrón generado por la oxidación de la prolina se escape del transporte de electrones mitocondrial y genere ROS (Lv et al., 2011).

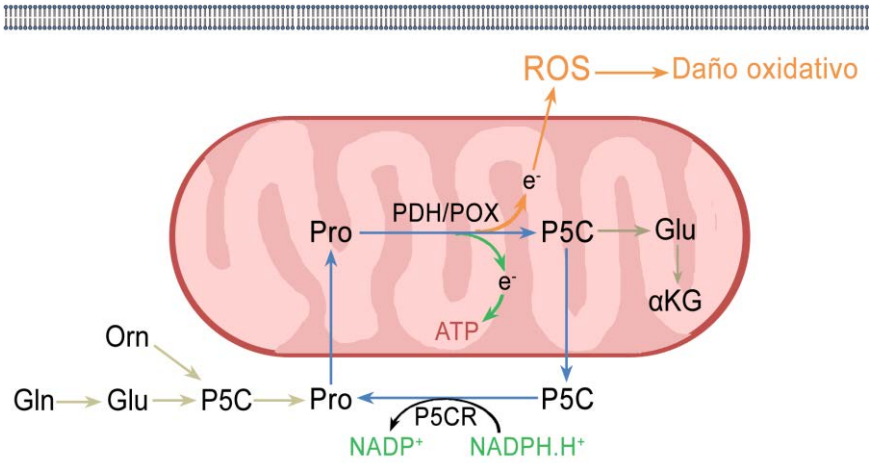

Figura 5. Ciclo Pro/P5C. El P5C rinde prolina en el citoplasma por acción de la P5CR. La prolina ingresa a la mitocondria por su trasportador o por el trasportador de glutamato, y es oxidada a P5C. El poder reductor que genera esta oxidación puede entrar en la cadena de trasporte electrónico, rindiendo ATP, o salir de la cadena favoreciendo la formación de ROS. El P5C puede salir de la mitocondria (trasportador aún no identificado en plantas) y rendir nuevamente prolina. αKG, α-cetoglutarato; Orn, ornitina; PDH, Prolina deshidrogenasa; POX, prolina oxidasa; P5CR, P5C reductasa.

Debido a que el rol protector de la prolina como antioxidante aún tiene un componente especulativo alto, y no se avanzó en cómo la prolina podría reaccionar con especies reactivas ni los productos derivados de su oxidación, en esta tesis se

realizaron estudios *in silico* e *in vitro* con el fin de inferir la funcionalidad de esta molécula en relación a su capacidad antioxidante.

Por otra parte, en especies de uso agronómico interesa conocer las respuestas frente al estrés nitro-oxidativo asociado a la sequía, ya que estas contribuirán con la tolerancia a esta condición. De esta forma, determinar la magnitud del estrés nitro-oxidativo generado por déficit hídrico y si la prolina tiene capacidad protectora frente a ROS/RNS, agregará evidencias sobre la habilidad que tienen las plantas de responder a la sequía.

OBJETIVOS

Objetivo general

Establecer respuestas bioquímicas que expliquen la tolerancia-sensibilidad de las plantas a la sequía.

Objetivos específicos

1. Identificar respuestas bioquímicas y/o fisiológicas en leguminosas forrajeras que expliquen la diferente tolerancia a sequía.

2. Determinar la magnitud del estrés nitro-oxidativo generado por sequía en diferentes órganos de la planta.

3. Establecer si la prolina tiene capacidad de proteger frente a diferentes especies reactivas del oxígeno y nitrógeno.

Capítulo 1. Respuestas a sequía de leguminosas

I. Evaluación de respuestas a sequía y calor mediante el uso de leguminosas forrajeras con tolerancia estival contrastante

Las respuestas mencionadas en la Introducción están bien documentadas para diversas especies de plantas sometidas a un solo tipo de estrés. Sin embargo el efecto producido por la combinación de estreses ha sido escasamente evaluado. En esta sección, que trata sobre el estrés combinado en leguminosas, se presenta un artículo (Signorelli et al., 2013*a*) y un capítulo de libro (Signorelli et al., 2015).

En general la sequía se establece principalmente en verano, por lo que las altas temperaturas acompañan al déficit hídrico en el establecimiento del estrés. En el artículo que se presenta a continuación se evaluaron las respuestas producidas por la combinación de déficit hídrico y alta temperatura en *Lotus corniculatus* (lotus) y *Trifolium pratense* (trébol), leguminosas forrajeras ampliamente utilizadas en praderas para alimentar ganado. Si bien trébol es más productivo que lotus, éste tiene mayor tolerancia estival, por lo que estas leguminosas con características diferentes se usaron como contrastantes en la respuesta a sequía y calor. Las plantas se sometieron a la combinación de déficit hídrico y alta temperatura (1 h a 42 °C) durante 5 días, para simular las condiciones de verano, y se midieron parámetros bioquímicos y fisiológicos con el fin de explicar la diferente tolerancia-sensibilidad.

Los resultados mostraron que estas leguminosas responden diferente en el control de la pérdida de agua, la acumulación de prolina, la capacidad antioxidante enzimática y la funcionalidad del fotosistema II. En lotus, a diferencia de trébol, en condición de sequía se indujeron las enzimas Mn-SOD y Fe-SOD. A su vez en trébol la actividad SOD total fue inferior a la de lotus. En este último la actividad fotosintética fue afectada por la combinación de estreses, lo que sugiere un proceso de foto-inhibición que se acompañó de la degradación de la proteína D2. Nosotros proponemos que descender la actividad fotosintética sin entrar en marchites permanente puede ser una ventaja de lotus en la disipación de la energía lumínica no utilizada y en la economía del agua, lo que le confiere más tiempo para una eventual rehidratación y

así una mayor tolerancia.

Por otra parte la sequía y/o el calor indujeron la acumulación de prolina en lotus, la especie más tolerante, mientras que trébol acumuló prolina sólo cuando la sequía se estableció en ausencia de calor. En la medida que con estrés combinado lotus acumuló prolina y toleró un día más el estrés que trébol, que no acumuló, podría hacer pensar que la acumulación de prolina confiere tolerancia al estrés combinado. Sin embargo otros aspectos como la diferente área foliar, conductancia estomática, actividad del fotosistema II, entre otras, pueden explicar diferencias en la tolerancia-sensibilidad. De esta forma, es demasiado especulativo explicar la tolerancia-sensibilidad a partir de la acumulación diferencial de prolina.

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Research article

Antioxidant and photosystem II responses contribute to explain the drought–heat contrasting tolerance of two forage legumes

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ABSTRACT

Identification of metabolic targets of environmental stress factors is critical to improve the stress tolerance of plants. Studying the biochemical and physiological responses of plants with different capacities to deal with stress is a valid approach to reach this objective. *Lotus corniculatus* (lotus) and *Trifolium pratense* (clover) are legumes with contrasting summer stress tolerances. In stress conditions, which are defined as drought, heat or a combination of both, we found that differential biochemical responses of leaves explain these behaviours. Lotus and clover showed differences in water loss control, proline accumulation and antioxidant enzymatic capacity. Drought and/or heat stress induced a large accumulation of proline in the tolerant species (lotus), whereas heat stress did not cause proline accumulation in the sensitive species (clover). In lotus, Mn-SOD and Fe-SOD were induced by drought, but in clover, the SOD-isoform profile was not affected by stress. Moreover, lotus has more SOD-isoforms and a higher total SOD activity than clover. The functionality and electrophoretic profile of photosystem II (PSII) proteins under stress also exhibited differences between the two species. In lotus, PSII activity was drastically affected by combined stress and, interestingly, was correlated with D2 protein degradation. Possible implications of this event as an adaption mechanism in tolerant species are discussed. We conclude that the stress-tolerant capability of lotus is related to its ability to respond to oxidative damage and adaption of the photosynthetic machinery. This reveals that these two aspects should be included in the evaluation of the tolerance of species to stress conditions.

1. Introduction

1.1. Biochemical responses to drought

Lotus corniculatus (lotus) and *Trifolium pratense* (clover) are legumes used in agriculture as a forage source. These species are both perennial herbaceous plants used in temperate grassland and can be nodulated by rhizobia. Nevertheless, lotus is better suited to soils with water restriction and has a superior tolerance to drought [1].

In the field, mainly during the summer, these plants are commonly exposed to environmental stresses such as drought and high temperatures, which in fact are considered to be the most important environmental factors limiting plant growth and development [2–5].

Soil water deficits resulting in dehydration and osmotic stress may seriously affect plant growth [6]. In response to a water deficit, plant cells accumulate low-molecular-mass compounds termed compatible solutes, mainly proline, glycine betaine, sugars and polyols, in the cytoplasm to accommodate the ionic balance in the vacuoles [7]. Among these solutes, proline has been associated with different functions, such as being a free radical scavenger, a cell redox balancer, a cytosolic pH buffer and a stabilizer for subcellular structures, especially during osmotic and salt stresses [8–10].

The accumulation of proline is known to be a good indicator of drought in *L. corniculatus* [11]. However, some plants that tend to accumulate proline in drought conditions replace it with sucrose as

Abbreviations: APX, ascorbate peroxidase; BSA, bovine seroalbumin; CAT, catalase; C, control treatment; D, drought treatment; D + H, drought and heat treatment; GR, glutathione reductase; H, heat treatment; HI, hydric index; H_2O_2, hydrogen peroxide; hydroxyl radicals, lotus; *Lotus corniculatus*, Pro; proline, P5C; pyrroline 5 carboxylate, PSII; photosystem II, ROS; reactive oxygen species, superoxide radical; SOD, superoxide dismutase; clover, *Trifolium pratense*.

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the major osmoprotectant when subjected to a combination of drought and heat stress [12]. It is also known that proline accumulation under heat stress decreases the thermotolerance of the plant, probably because of an enhancement in the production of reactive oxygen species (ROS) via the Pro/P5C cycle [13]. Additionally, drought affects the rate of photosynthesis due to an increase in photoinhibition, a process that can be enhanced when two or more types of abiotic stress coexist [14].

Under stress conditions, the possibility of overexcitation of photosystem II (PSII) increases. This can cause a decline in the photosynthetic rate as the process of photoinhibition increases due to the necessity to dissipate, through nonradiative processes, the excess of absorbed energy [14,15].

Because the capacity of photoprotection is limited, certain conditions can lead to damage and loss of active PSII reaction centres. Under severely high temperatures, stress commonly associated with a water deficit during drought, the photosynthetic apparatus has been long considered to be the primary site of damage. On the contrary, photosystem I has been shown to be more resistant to heat than PSII [16–18]. Once photoinhibition is established, the PSII reaction centre is simultaneously repaired via removal, synthesis and replacement of degraded D1 protein [19,20]. The observed photoinhibitory damage is the net result of a balance between photodamage and the repair process [21–23].

Several studies have reported a good correlation between changes in chlorophyll fluorescence parameters in response to environmental stresses, such as heat, chilling, freezing, and salinity [24–27]. Others authors have linked the decrease in the maximum quantum yield of PSII (F_V/F_M) to the physical dissociation of the PSII reaction centres that lead the photoinhibition, and they performed this technique to discriminate tolerant cultivars [28].

It is well known that the effect of a combination of different stresses on plants can be quite different from those generated when plants are subjected to individual types of stress [29], and is motive of studio.

1.2. Water–heat stress and ROS metabolism

Another effect of water and heat stress is an overproduction of ROS, such as hydrogen peroxide (H_2O_2), superoxide radicals ($O_2^{\bullet -}$) and hydroxyl radicals ($^{\bullet}OH$), which occurs mainly in organelles such as the chloroplast, mitochondria and peroxisomes [30,31]. These species are responsible for lipid and protein oxidation, the primary consequences of ROS-mediated oxidative stress [32]. This stress can cause damage to the PSII, mainly when it occurs at the chloroplast level, leading to disassembly of the different structures through proteolysis.

In particular, the D1 protein is damaged because of intrinsic ROS generation by the PSII machinery [33], which can also impair the activity of the Calvin cycle and disrupt electron transport [34]. This situation generates metabolic limitations and, together with the stomatal restriction generated during drought conditions, leads to a decay in carbon assimilation [35–37].

The enzymatic antioxidant systems include superoxide dismutase (SOD; EC 1.15.1.1), which provides the first line of defence against ROS by dismutating $O_2^{\bullet -}$ to H_2O_2, and catalase (CAT; EC 1.11.1.6), which regulates H_2O_2 levels [30]. These enzymes, in combination with enzymes of the ascorbate–glutathione cycle, such as ascorbate peroxidase (APX; EC 1.11.1.11) and glutathione reductase (GR; EC 1.6.4.2), protect plant cells by reducing the generation of toxic oxygen forms. A number of studies have shown that the antioxidant defence systems are induced and up-regulated to a greater extent in water stress-tolerant cultivars compared to sensitive species such as apple [38], tomato [39] and alfalfa [40] when subjected to water stress, and the induction of these systems is associated with acclimation to water deficit conditions. The generation of oxidative stress induced by abiotic stress and mediated by ROS accumulation is widely documented [30]; however, additive or synergistic effects of two or more environmental stresses on ROS-related photoinhibition of PSII are poorly understood.

According to Boyer (1982) [6], water shortages limit crop production in semi-arid regions more than any other factor. A better understanding of the mechanisms that enable plants to adapt to water deficits and maintain growth and productivity during drought periods will ultimately help in the selection of drought-tolerant varieties.

Studies indicate that even though drought and heat stress can occur together in most regions (particularly the semi-arid tropics), the physiological or biochemical mechanisms operating to induce escape or tolerance to each of these stresses may be different. Furthermore, the characteristics of the traits associated with drought and heat stress might be different. Therefore, the selection–production of genotypes for tolerance to combined drought and heat stress must be performed under stress conditions that include both of these stresses [41].

Lotus and clover have different tolerance to drought and drought–heat combination, stressing environmental conditions frequently found during the summer. We sought to explain the differences in tolerance through the evaluation of the antioxidant and PSII responses of leaves under stress conditions to contribute to the understanding of differential field drought tolerance in two forage legumes.

2. Results

2.1. Differential response to dehydration induced by drought

To confirm the differential behaviour observed between lotus and clover when they are subjected to drought, we first followed

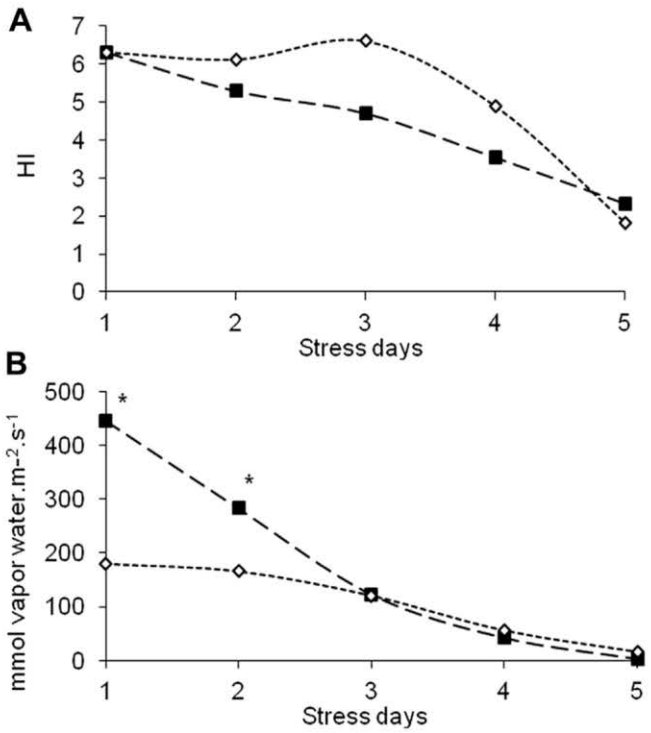

Fig. 1. Hydric index and stomatal conductance. A. Hydric index (HI) in the 5 days of treatments. B. Stomatal conductance during the 5 days of treatments. Lotus is indicated by white diamonds (◇) and clover by black squares (■). Asterisks indicate that differences in the clover values compared to the lotus values were statistically significant at $p \leq 0.05$, Tukey's test.

the water status of both species after the withdrawal of watering (Fig. 1A). The data show that clover leaves start to lose water after one day of treatment, and this continued during the next four days. However, lotus did not show evident loss of water during the first three days, and the water losses began after the third day (Fig. 1A). Stomata conductance was measured during the drought stress (Fig. 1B), and different patterns were also found in these species. It is important to note that basal conductance under control conditions was significantly different between the species. Therefore, while stomata closure was observed in clover after one day of drought, in lotus three days of drought were necessary to detect changes in stomata conductance (Fig. 1B).

Data obtained by imposing drought conditions confirm previous evidence on differences in drought tolerance observed in this species under summer field drought conditions.

However, differences between these forage legume species cannot be explained only by water status maintenance. Thus, others environmental factors should be involved in the summer drought tolerance/susceptibility response in plants, such as heat stress. In this case in particular, the drought–heat stress combination might be a key factor to analyse for proper assessment of plant stress behaviour.

2.2. Lotus and clover phenotypes under drought–heat stress

Fig. 2 shows the phenotypes of lotus and clover plants at the end of each treatment. Drought produces an evident loss of turgor in both species, but plants exposed to heat treatment did not present any visual differences compared to control plants (well-irrigated conditions). Combined treatment (drought + heat) also produced a loss of turgor in both species. However, in clover, this loss was accelerated by heat, resulting in a withered appearance one day before lotus. For this reason, clover in the combined treatment was photographed on the fourth day because on the fifth day, the clover plants were already dead. Since our goal was to assess the oxidative and photosynthetic responses to severe stress, and not at a specific time after the imposition of the stress condition, analyses were made in plants with similar water content (Fig. 2). Therefore, all of the determinations in clover in the combined treatment were performed on the fourth day, although the other treatments in both species ended on the fifth day.

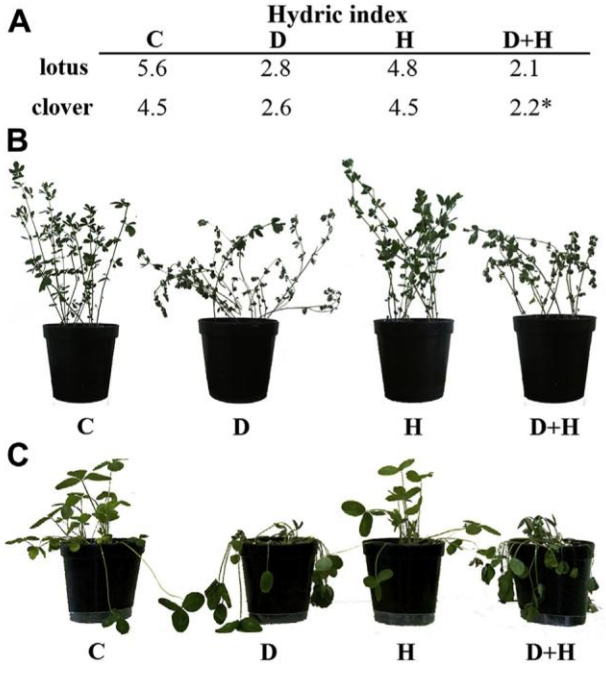

Fig. 2. Lotus and clover plant phenotypes and hydric index in the different treatment conditions. A. Hydric index. B. Phenotypes of lotus plants. C. Phenotypes of clover plants. C, control; D, drought; H, heat at 42 °C; D + H, drought + heat at 42 °C. Determinations were made on the last day (5) of each treatment except for *, which was measured on the fourth day of treatment.

2.3. Free proline accumulation as a stress response indicator

The free proline concentration was measured in leaves at the end time of each treatment (Fig. 3). Lotus accumulated proline in the drought and heat treatments, but concomitant imposition of both stresses produced a higher accumulation of the aminoacid. In contrast, clover accumulated proline in drought conditions but not under heat stress, and the imposition of the combined treatment produced only a slight increase in proline concentration.

2.4. Antioxidant enzymes and ROS metabolism

2.4.1. SOD isoforms activities and total SOD activity

The activity of SOD isoforms was evaluated in a native gel with specific inhibitors (Fig. 1S). Lotus has five SOD isoforms, including Mn-SOD, Fe-SOD and three Cu/Zn-SOD, while clover has six isoforms, including two Mn-SOD, one Fe-SOD and three Cu/Zn-SOD (Fig. 4A and Fig. 1S). In lotus, the activity of Mn-SOD and Fe-SOD increased as a consequence of drought treatment, but it did not change in the heat and combined treatments. In clover, however, no changes were observed in the activities of any SOD isoforms. In clover, the activity of SOD with 40 μg of protein could not be clearly detected (data not shown), and for this reason we used more protein from clover than from lotus to visualise the SOD isoforms in both species (Fig. 4A). This result suggested that the total SOD activity is higher in lotus than in clover, and for this reason we quantified the total SOD activity in control and stressing conditions (Fig. 4B). The results of the quantitative enzyme activity assay confirmed that total SOD activity is 2.6 folds greater in lotus than in clover and it is affected by the stress treatments. Drought induced the activity in both legumes species; however, lotus showed a significantly increase in SOD activity respect to clover. Heat did not modify the SOD activity in lotus, but the combination with drought led the activity to same level observed in the drought treatment (Fig. 4B). Clover showed a slight increase in the SOD activity only those treatment involving heat stress (Fig. 4B).

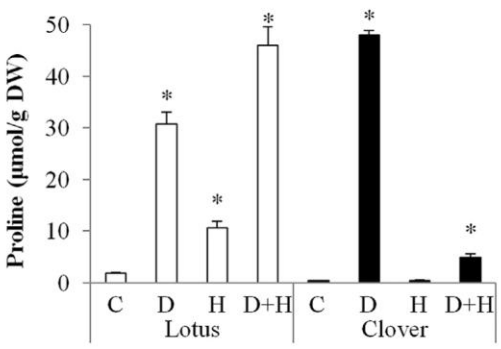

Fig. 3. Proline concentration in leaves of lotus and clover. C, control; D, drought, H, heat; D + H, drought and heat. Data are means ± SD of at least three biological replicate measurements. Asterisks indicate that differences from control values were statistically significant at $p \leq 0.05$, Tukey's test.

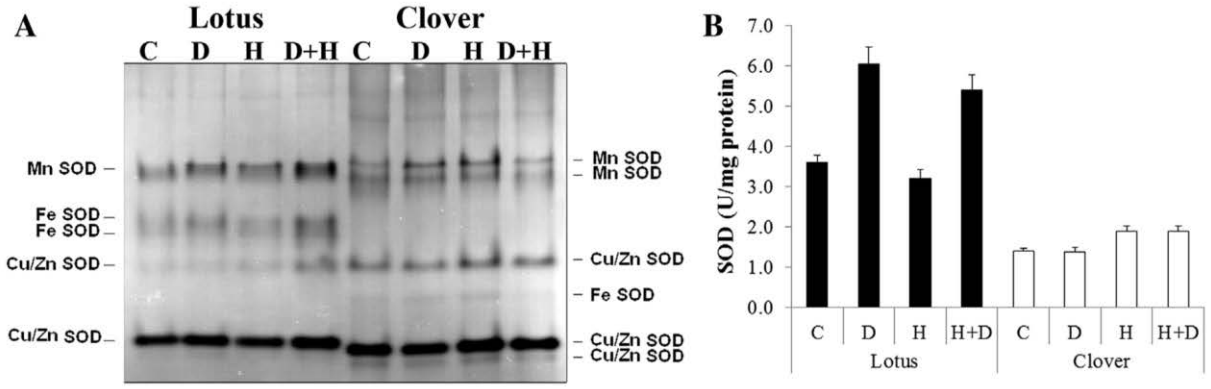

Fig. 4. SOD activity in under drought and heat–drought stress combination. A. SOD isoforms profile. C, control; D, drought; H, heat at 42 °C; D + H, drought + heat at 42 °C. 40 μg and 200 μg of protein were loaded in lotus and clover respectively. The gel is the most representative of three replicates of native-gels. B. Total *in vitro* SOD activity. C, control; D, drought; H, heat at 42 °C; D + H, drought + heat at 42 °C. One unit of SOD was defined as the amount of enzyme which inhibits the rate of cytochrome c reduction by 50%. Bars indicate the relative standard deviation (RSD).

2.4.2. Catalase and ascorbate–glutathione cycle

In lotus, catalase activity only increases in the combined treatment. However, in clover, enzyme activity increased with respect to the control in all of the treatments, although no differences were observed in between these treatments (Fig. 5). In control conditions, APX and GR activities in clover were half of those observed in lotus. The APX activity in lotus was inhibited by drought condition, while in clover this activity was inhibited only in the stress combined treatment (Fig. 5). The GR activity in lotus was negatively affected under drought and heat stress conditions (Fig. 5), however, in clover this enzyme activity was not affected by the stress treatments (Fig. 5).

2.4.3. $O_2^{\bullet-}$ and H_2O_2 accumulation

We used a qualitative technique to detect the accumulation of $O_2^{\bullet-}$ and H_2O_2. Both lotus and clover leaves showed staining associated with $O_2^{\bullet-}$ accumulation only in the drought–heat combination (Fig. 6). In clover, H_2O_2 accumulation showed the same pattern; however, in lotus, the highest staining for this molecule was observed under drought conditions. Since the alterations found in CAT, APX and GR activities under stressful conditions we quantified the H_2O_2 contents using the Amplex red® kit. Increments of H_2O_2 concentration seem to occur in lotus and clover under drought and combined treatment, respectively, which correlate with the staining method (Fig. 6). However, these values did not differ statistically, probably because of the low reproducibility of this technique.

2.4.4. Lipid peroxidation

Lipid peroxidation was measured by thiobarbituric reactive substances (TBARS). Lotus showed an increase in TBARS content in response to all treatments (Fig. 7). Similar results were observed in clover, with the exception of the heat treatment, which did not produce any increase in TBARS content.

2.5. Photosynthesis stress response

2.5.1. Maximum quantum efficiency

In order to determine the effects of drought and heat stress on the photosynthetic activity of lotus and clover, we measured the maximum quantum efficiency, evaluated as F_V/F_M, obtained from certain chlorophyll fluorescence parameters. F_V/F_M was monitored during the five days of treatment (Table S1). Table 1 shows the summarised data including the first and the last day of each treatment. In lotus, no changes were observed in any treatment until the fifth day, when the combined treatment showed a significant decrease in the F_V/F_M parameter (Table 1). In contrast, in clover, this fluorescence parameter slightly decreased from the first day in the heat and combined treatment, but no changes were observed under drought conditions (Table S1). This ratio could not be determined on the fifth day in the combined treatment because the plants were dead.

2.5.2. Photosystem II proteins

The chloroplastic D1 and D2 proteins were studied to evaluate degradation induced by drought, heat and the combination of both stresses. The chloroplastic protein profile and the western blot analyses of D1 and D2 are shown in Fig. 8. Lotus showed a slight decrease in the amount of D1 protein after drought treatment. However, there was no decrease in the protein content when the control and heat conditions were compared. The D1-complex profile of clover was also analysed, and the western blot showed a very different result when was compared to the lotus profile. The total D1 protein content in clover did not change in any treatment, but a difference was found in the ratio between the free protein and the complex form. In the treatments where heat was involved, an increase in free D1 protein together with a decrease in the D1-complex form was evident, but it should be considered that this result might be a consequence of the high hydrophobicity of these complexes, which makes their isolation difficult.

Regarding D2, in lotus the results were similar to those observed with D1; namely, when drought was present in the treatments, a reduction in the amount of D2 protein was observed. Surprisingly, in the combined treatment, the D2 protein was not detected. Another western blot with more loaded protein and a higher exposure time of the radiographic film was needed to even barely detect the complexes and D2 protein in the combined treatment (Fig. S2). In contrast, in clover, no significant changes were observed in D2 protein levels.

3. Discussion

3.1. Drought–heat phenotype

Is well known that metabolic strategies to deal with environmental abiotic stress vary by plant species and genotype [42]; however, the physiological and biochemical differences that determine contrasting seasonal drought tolerance in forage legumes are poorly understood. As reported here, lotus and clover, two forage legumes with contrasting drought tolerances [1], have

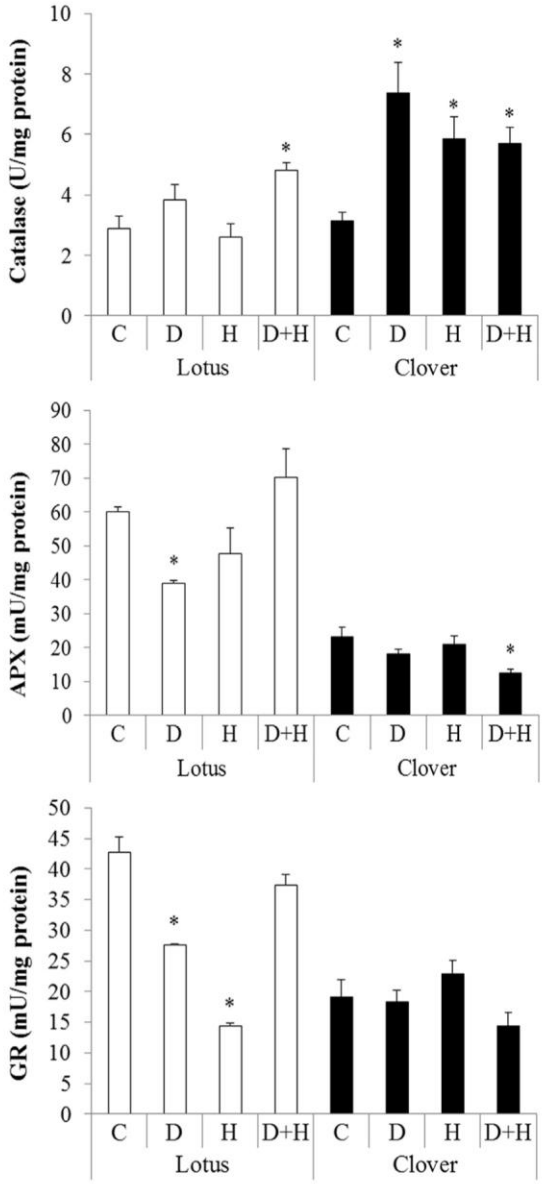

Fig. 5. CAT, APX and GR activities. C, control; D, drought; H, heat at 42 °C; D + H, drought + heat at 42 °C. Data are means ± SD of at least three biological replicate measurements. Asterisks indicate that differences from control values were statistically significant at $p \leq 0.05$, Tukey's test.

different capacities to control water loss, and stomatal conductance was shown to be one, but not the only, differential response between the legumes. In this way, stomatal conductance is related to drought tolerant-sensitive responses in several plant species [43,44].

In this work, we propose that, for lotus, proline accumulation is a parameter that should be used as a stress marker to assess drought and heat stress conditions, as well as the combination of both. In clover, however, this parameter is useful under drought conditions, but not during a drought–heat combination. In this species, our results are in accordance with Rizhsky et al. (2004) [12], who showed that proline accumulation is not a suitable factor for use in a combined stress situation.

Taking into account that the accumulation of proline during heat stress (50 °C) increases ROS production in *Arabidopsis thaliana* [13], blocking proline accumulation might be a strategy for avoiding self-toxicity in heat-tolerant species. In our study, this hypothesis

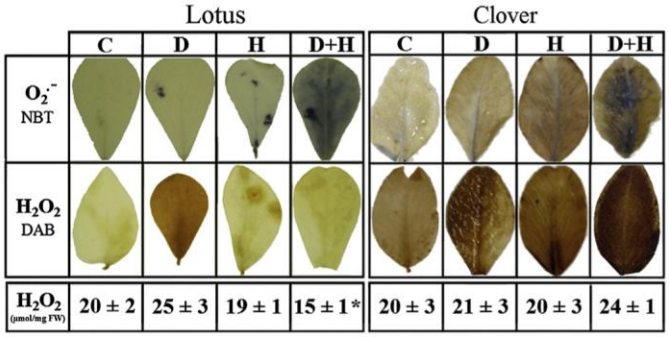

Fig. 6. $O_2^{\cdot-}$ and H_2O_2 accumulation. NBT and DAB staining are evidence of the presence of $O_2^{\cdot-}$ and H_2O_2 respectively. Images are the most representative of a pool of five leaves in each treatment. Concentration of H_2O_2 in leaves is expressed in μmol/mg FW. Data are means ± SD of at least three biological replicate measurements. Asterisks indicate that differences from control values were statistically significant at $p \leq 0.05$, Tukey's test. C, control; D, drought; H, heat at 42 °C; D + H, drought + heat at 42 °C.

correlates with the lipid peroxidation estimated by TBARS, as clover does not show an increase in lipid peroxidation under heat conditions, in contrast to lotus. Moreover, clover has a lower lipid peroxidation content than lotus when drought and heat stress are combined, a treatment in which lotus accumulates the highest levels of proline.

On the other hand, it has been demonstrated that proline can act as an osmolyte under severe dehydration [45]. The non-accumulation of proline and the greater leaf area in clover are important disadvantages of this species compared to lotus when water loss must be prevented. Clover did not survive to the fifth day under the combined stress treatment, while lotus was still alive on that day. Thus, lotus is more tolerant to drought when this stress is established in a high temperature condition. In agreement with our results, a higher dry matter yield was observed in lotus with respect to clover under field conditions subjected to summer drought [1].

3.2. ROS metabolism

Adaptation to drought may depend on different mechanisms, including the capacity to maintain high levels of antioxidants and to regenerate them through the induction of detoxification enzyme activities [46]. Moreover, a previous report on *Lotus japonicus* showed that drought induces mainly oxidative stress in the photosynthetic tissues [47]. Our results clearly show that lotus has a higher response of SOD-dependent antioxidant machinery

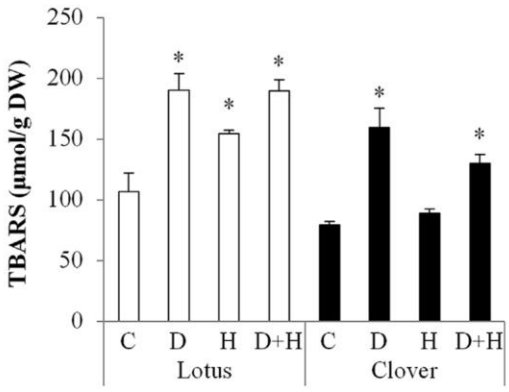

Fig. 7. TBARS concentration. C, control; D, drought; H, heat at 42 °C; D + H, drought + heat at 42 °C. Data are means ± SD of at least three biological replicate measurements. Asterisks indicate that differences from control values were statistically significant at $p \leq 0.05$, Tukey's test.

Table 1
Heat and drought effects on the maximum quantum efficiency (F_V/F_M) of lotus and clover. C, control; D, drought; H, heat at 42 °C; D + H, drought and heat at 42 °C. Values are means ± SD of at least three biological replicate measurements.

Treatment	Day	Lotus	Clover
		F_V/F_M	F_V/F_M
C	1	0.87 ± 0.01	0.88 ± 0.01
	5	0.87 ± 0.02	0.86 ± 0.01
D	1	0.86 ± 0.01	0.86 ± 0.01
	5	0.86 ± 0.01	0.85 ± 0.02
H	1	0.85 ± 0.02	0.83 ± 0.03
	5	0.85 ± 0.02	0.77 ± 0.04
D + H	1	0.88 ± 0.02	0.82 ± 0.03
	4	0.82 ± 0.03	0.79 ± 0.02
	5	0.47 ± 0.15	[a]

[a] Without data because plants were dead.

because this species has higher basal activity compared with clover and also the Mn-SOD and Fe-SOD increased under drought condition. SOD is the main enzymatic system responsible for cell detoxification and is well documented in several plant species to increase in response to hydric deficit and heat stress [48,49]. *In situ* detection of $O_2^{\bullet-}$ in lotus leaves showed that combined drought and heat induced significant accumulation of this radical, as was previously observed in the model legume *L. japonicus* [50]. In clover, accumulation of $O_2^{\bullet-}$ was observed in all stress treatments and increased even more in the combined treatment. The higher SOD activity in drought conditions with respect to controls (Fig. 4), would allow this species to deal with the $O_2^{\bullet-}$ induced mainly by drought (Fig. 6). However, the increase of Mn-SOD and Fe-SOD isoform activity by drought was lost under high-temperature conditions (Fig. 4), resulting in an increase of $O_2^{\bullet-}$ in the combined treatment (Fig. 6). In *L. japonicus* similar results were obtained with Cu/Zn-SOD, showing that deleterious effects of heat stress on SOD activity might be a general response for this legume genus [50]. Moreover, importantly the differences detected between both species are mainly explained by changes in the Cu/Zn-SOD isoforms.

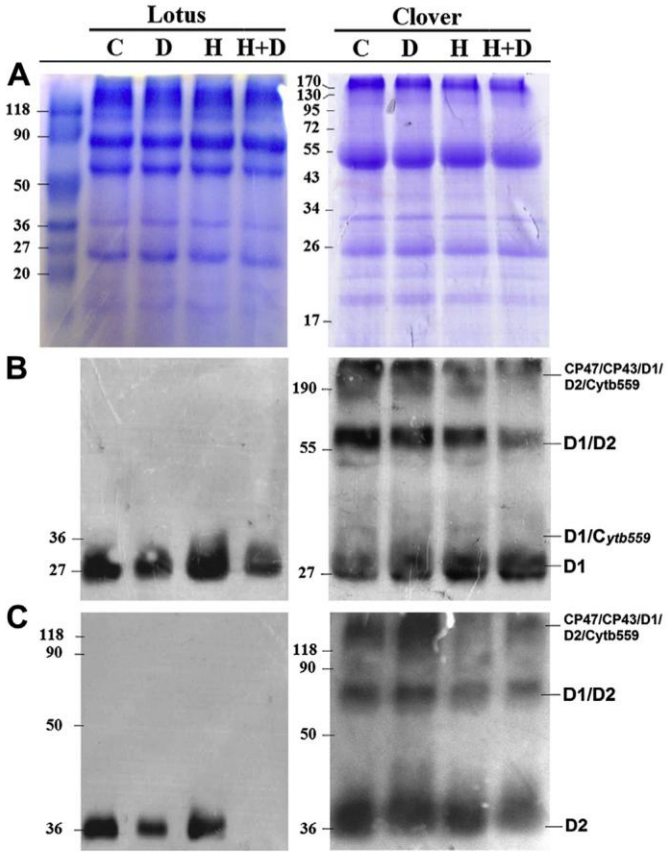

Fig. 8. Photosystem II proteins of lotus and clover in the different treatment conditions. A. Chloroplastic isolated protein profile. B. D1 protein detection by western blotting. C. D2 protein detection by western blotting. Western blots were made loading 20 μg of chloroplastic protein of each treatment. C, control; D, drought; H, heat at 42 °C; D + H, drought + heat at 42 °C. Numbers at the left indicate the corresponding kDa of the molecular weight marker.

The increase in the H_2O_2 levels observed in lotus as a consequence of the drought conditions is related to APX and GR activities, as these enzymes activity decreased in this stress condition, and CAT was not induced. However, the increase in the activity of CAT enzyme observed in the combined treatment (Fig. 5) was enough to keep the basal levels of H_2O_2 under control (Fig. 6). In clover, although CAT activity increased in all treatments the other peroxidase APX and the GR did not respond to the stress conditions. Even more, a reduction in the APX activity in the combined treatment resulted in the accumulation of H_2O_2, since we detected the presence of this ROS by the staining method in the stress combined condition (Fig. 6). The H_2O_2 determination with commercial kits, like Amplex red®, has limitations mainly assigned to the extraction of H_2O_2, which are causes of problems in the reproducibility of this method (for revision see Ref. [51]). We considered that an important limitation of these techniques is the non-linear correlation between the amount of tissue extract used and the contents determined. This does not allow discriminating statistically the different treatments. For this reason we think that staining with DAB, a semi-quantitative method which avoid the extraction of H_2O_2 from the tissue, is still useful.

In drought-sensitive wheat, the increased capacity to metabolise H_2O_2 by detoxification enzymes has been reported [46], suggesting that responsiveness of these enzymes is not a guarantee of drought stress tolerance.

Regarding lipid peroxidation, it is notable that in both species, increases in the levels of either $O_2^{\bullet-}$ or H_2O_2 that occur in the treatments involving drought are associated with increases in TBARS levels, a fact that is in accordance with many reports [52,53].

In lotus, an increase in lipid peroxidation is also observed during heat stress, while no damage was observed in clover under the same stress condition. This increase in lotus correlates with an increase in proline concentration, which could result a higher ROS production by the Pro/P5CS cycle, leading to major oxidative damage, as previously mentioned [13]. In this way, proline may play a critical role under drought-induced osmotic stress and confer an adaptive advantage to lotus, but it can provoke a deleterious effect when a heat stress condition is present. As proline antioxidant protection function on chloroplasts under stress conditions is now in discussion [54], the absence of proline accumulation in clover becomes an advantage under heat stress by avoiding the Pro/P5CS cycle. However, proline accumulation might be critical under combined stress because the osmolyte function seems to be important when drought is established. Whether proline accumulation is a positive or negative trait in regards to drought tolerant genotypes remains to be elucidated.

3.3. Photosystem response

F_V/F_M, the chlorophyll fluorescence parameter evaluated, showed that lotus had an important decrease in the maximum quantum efficiency at the end of the combined treatment. The low F_V/F_M ratio indicated the presence of photoinhibition, a process that can act as determinant for whether the plant will have a better

performance during a stress condition [28]. In clover, only a small decrease in the F_V/F_M values was observed from the first day and in the treatments involving heat stress.

Analysis of photosystem II proteins in the two drought-contrasting legumes shows an effect that is stress- and species-specific. The D1 and D2 subunit content is decreased in lotus in both treatments involving drought (Fig. 8), showing certain adaptability in response to drought. Interestingly, the decrease in the D2 levels was pronounced in the combined treatment (Fig. 8), and this is well correlated with the decrease in the maximum quantum efficiency, suggesting the presence of a disassembling process. The D2 subunit is of particular interest because it represents the initial point for the assembly of the PSII as a whole [55–57]. It has been found that the expression of the gene which encodes the D2 subunit of the PSII reaction centre is regulated post-transcriptionally by an RNA-binding protein able to affect translation initiation of the D2 protein [58]. Modifications induced by the stress in this post-transcriptional regulation could be a possible explanation of the D2 absence in lotus subjected to the combined stress treatment.

In clover, the total content of D1 and D2 did not change, but we observed an increase in the free form of D1 in treatments involving heat (Fig. 8). One possible explanation is that turnover of D1 is taking place in the heat treatments, and this is evident by an increase of free D1 together with a reduction of the D1–D2 complex, as well as a decrease in the F_V/F_M values (Table 1).

3.4. Photosynthetic adaptation and the antioxidant system are responsible of differential tolerance to drought in forage legumes

Lotus and clover, two legumes with contrasting drought tolerance showed clear differences in their responses to dehydration. Water loss in clover was not accompanied by changes in SOD and GR activities, F_V/F_M or PSII proteins, resulting in earlier dead plants. In contrast, in lotus, the tolerant species, water loss is more controlled and significant responses were observed in all of the studied parameters, with a magnified response in the drought–heat stress conditions. In this way, our work is the first report in which a drought-tolerant species decreases its photosynthetic activity, evident by F_V/F_M, due to a decline of the D2 protein of the PSII reaction centre in response to drought–heat stress.

Several studies have shown a drought-dependent decrease in photosynthetic activity by alterations in the corresponding biochemical components and processes, with PSII being the most sensitive of these components to drought stress [59].

Loggini et al. (1999) suggested that two drought-tolerant wheat cultivars had different cellular mechanisms to cope with water stress. We focused on key elements that might be responsible for drought tolerance. The study of the response of plants to drought stress has only been reported in a few studies that showed that this response is likely to be of fundamental importance for tolerance [43,46,59]. Taking into account our results, we can conclude that the tolerant capability of lotus is strongly related to its ability to sense stress and adapt to it, as in its response to oxidative damage and adaption of its photosynthetic machinery. This reveals that these two aspects should be included in the evaluation of the tolerance of species to stress conditions, especially with a combined treatment of drought and heat.

4. Materials and methods

4.1. Plant material and growth conditions

L. corniculatus cv. San Gabriel and *T. pratense* cv. LE116 seeds were obtained from M. Rebuffo INIA La Estanzuela (Colonia, Uruguay). The seeds were surface sterilised as described by Orea et al. [60] and germinated at 28 °C for 2 days. Seedlings were transferred to pots containing a mix of river sand:vermiculite (1:1) as substrate, and plants were grown for 35 days under controlled conditions: 16/8 h light/dark cycle at 24/18 °C with a photosynthetic photon flux density of 200 μmol m^{-2} s^{-1}. Plants were irrigated daily with Hornum's nutrient solution [61]. Drought was induced by suspension of irrigation, and measurements were made over the following 4 days without irrigation. The heat stress treatment involved transferring the plants to a growth chamber at 42 °C for 4 h each day. Leaves were harvested at the end of each treatment, frozen quickly in liquid nitrogen and stored at −80 °C until analysed.

4.2. Protein extraction and quantification

Leaves were frozen in liquid N_2 and ground in a mortar. The powder was suspended with homogenizing-buffer composed of 50 mM Tris–HCl, pH 7.8, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol (DTT) and 0.2% (v/v) glycerol. The ratio powder:homogenizing-buffer was 1:2 for control and heat treatments and 1:3 for treatments wherein drought was involved, because the lower water contents in these samples. Homogenates were centrifuged at 20,000× *g* for 20 min (4 °C) and supernatant was used immediately after purification by gel filtration using PD-10 Sephadex® G-25M Columns, proteins were eluted with the 3 mL of homogenizing-buffer described above. Protein concentration of extracts was measured by the Bradford method [62] using BSA as standard.

4.3. Oxidative metabolism

4.3.1. Lipid peroxidation

Lipid peroxidation was estimated using the method proposed by Minotti and Aust [63]. Lipid peroxides were detected as thiobarbituric acid reactive substances (TBARS) by measurement of malonaldehyde absorbance at 532 nm, and their concentration was determined using an extinction coefficient of 156 mM^{-1} cm^{-1} [64]. Four replicates of each sample were assayed.

4.3.2. SOD isozyme identification and SOD total activity

Cytosolic and chloroplastic SOD (EC 1.15.1.1) isozymes were analysed on non-denaturing polyacrylamide gels, as described by Laemmli [65], but without SDS, using in-gel activity assays with isozyme-specific inhibitors previously described by Donahue et al. [66].

Total SOD activity was measured using the ferricytochrome c reduction assay, following the reduction rate of cytochrome c by $O_2^{\bullet-}$ at 550 nm, utilizing the xanthine–xanthine oxidase system as the source for $O_2^{\bullet-}$ as described by Flohe and Otting [67]. One unit of SOD was defined as the amount of enzyme which inhibits the rate of cytochrome c reduction by 50%. The curve was established from the data of activity in eight independent extracts with five replicates for each extracts. The curve was linearised by plotting the reciprocal absorbance change per minute versus the volume of extract and then the SOD activity was determined as described in Ref. [67].

4.3.3. CAT, APX and GR activities

All the enzyme assays were performed from protein extracts as described in 4.1.2. CAT (EC 1.11.1.6) activity was determined by measuring the disappearance of H_2O_2, as described by Aebi [68]. APX (EC 1.11.1.11) activity was determined by monitoring oxidation of ascorbate by H_2O_2 at 290 nm [69]. GR (EC 1.6.4.2) activity was assayed by monitoring oxidation of NADPH coupled to the reduction of GSH [70]. The reaction rate was corrected for the small non-enzymatic oxidation of NADPH by glutathione disulfide (GSSG).

One enzymatic unit was defined as the activity that consumed 1 μmol of cofactor or substrate per minute.

4.3.4. Superoxide and hydrogen peroxide determinations

In situ detection of superoxide ($O_2^{\bullet-}$) was performed as described by Jabs et al. [71]. Detached leaves were vacuum infiltrated with 10 mM potassium phosphate buffer pH 7.8, 10 mM NaN_3, 0.1% (w/v) nitro blue tetrazolium (NBT) and 0.05% (v/v) Tween 20. The detached, infiltrated and NBT-treated leaves were then maintained for 30 min under daylight conditions prior to discolouration of the leaves using the same method described above for the detection of H_2O_2.

In situ detection of H_2O_2involved following the protocols proposed by Thordal-Christensen et al. [72]. Detached leaves were vacuum infiltrated under dark conditions with 10 mM potassium phosphate buffer, 10 mM NaN_3 and 0.1% (w/v) 3,3′-diaminobenzidine (DAB), pH 7.8. Leaves were incubated overnight under dark conditions and then clarified with 0.15% (w/v) trichloroacetic acid in 4:1 (v/v) ethanol:chloroform for 48 h before being photographed.

For H_2O_2 quantification, 0.2 g of leaf tissue was homogenised in 1 mL of 0.2 M $HClO_4$ using a pre-cooled mortar. The homogenates were centrifuged three times at 4 °C (15 min 10,000× *g*) to further clarify the supernatant obtained in each case. The final supernatant obtained was neutralised to pH 7.0 with 0.2 M NH_4OH and centrifuged for 2 min at 3000× *g* to remove additional remaining insoluble material. Additional steps were done as described by Rao et al. [73]. H_2O_2 in the extracts was measured in triplicate using the Amplex Red Hydrogen Peroxide-Peroxidase AQ3 Assay kit® (Invitrogen-Molecular Probes) following the manufacturer's instructions.

4.4. Photosynthetic determinations

4.4.1. Chlorophyll fluorescence parameters

Chlorophyll fluorescence measurements were made on fully expanded leaves using an FMSI fluorometer (Hansatech Instruments Ltd., King's Lynn, UK) using saturating actinic light (8000 μmol m^{-2} s^{-1}) for 0.8 s. Leaves were dark adapted for 30 min prior to analysis. F_V/F_M for PSII was determined from chlorophyll fluorescence parameters, which were calculated as described by Rohácek and Barták [74].

4.4.2. Immunodetection of D1 and D2 proteins

Immunodetection of D1 and D2 was performed with cell-free protein extracts from isolated chloroplasts obtained using the CP-ISO kit (Sigma Chemical, St. Louis, MO) following the manufacturer's instructions. Aliquots containing 20 μg of protein from a given supernatant preparation were separated by electrophoresis on a 12% (w/v) sodium dodecyl sulphate (SDS) polyacrylamide gel [75]. The separated proteins were then electroblotted onto polyvinylidene fluoride (PVDF) membranes (Immobilon P, Millipore, Billerica, MA, USA), and rinsed with TBS (20 mM Tris–HCl; 0.5 M NaCl, pH 7.5). Western blot analysis was performed according to the manufacturer's instructions (ECL Plus Western AQ2 Blotting Detection System, Amersham®). Antibodies against D1 (AS05 08410) and D2 (AS06 146) were obtained from Agrisera (Vännäs, Sweden).

4.5. Other determinations

4.5.1. Hydric index

To determine the hydric index (HI), fresh leaves were weighed (FW) and then dried at 80 °C. The dry weight was then measured (DW). The HI was calculated as follows: FW/DW. HI, analytical and biochemical determinations were performed with pooled samples of leaves harvested at the indicated time [76].

4.5.2. Stomatal conductance

Stomatal conductance was measured using a Porometer Model SC-1 (Decagon Device) on the abaxial surfaces of six leaves of at least three independent plants as instructed by the manufacturer.

4.5.3. Proline quantification

Proline was extracted from 100 mg of leaves with a mixture of methanol–chloroform–water (12:5:1) as described by Charest and Phan [77] and quantified according to Borsani et al. [78].

4.6. Statistical analysis

Analysis of variance was performed with data from three independent experiments, and means from the results from experiments were compared using Tukey's test at the $p \leq 0.05$ level.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2013.05.028.

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Supplementary information

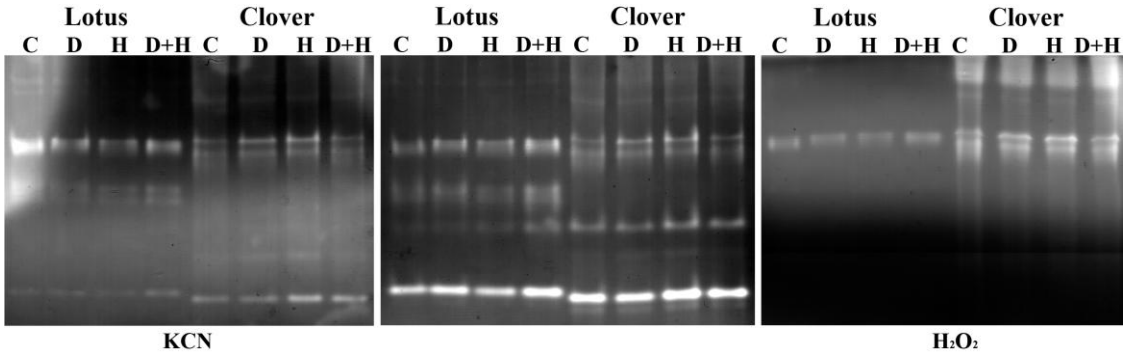

Figure S1. SOD isoforms profile under drought and heat-drought stress combination with (KCN and H_2O_2) and without inhibitors. C, Control; D, Drought; H, Heat at 42ºC; D + H, Drought + Heat at 42ºC. 40 µg and 200 µg of protein were loaded in lotus and clover respectively.

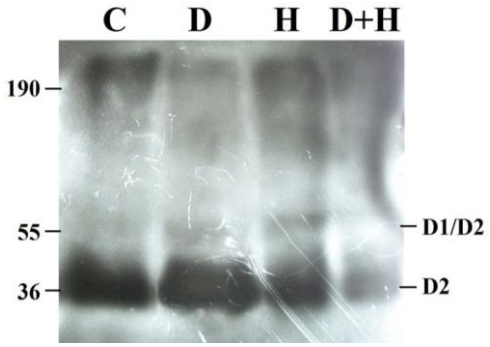

Figure S2. D2 proteins of lotus subjected to stress conditions. Western blots were performed by loading 30 µg of chloroplastic protein of each treatment. C, Control; D, Drought; H, Heat at 42 ºC; D + H, Drought + Heat at 42 ºC. Numbers on the left side indicate molecular weight in kDa.

Table S1.

Table S1. Heat and drought effects on the maximum quantum efficiency (F_v/F_m) of lotusand clover. C, Control; D, Drought; H, heat at 42 ºC; D + H, Drought and heat at 42 ºC. ND: No data. Values are means ± SD of at least three biological replicate measurements.

Treatment	Day	Lotus Fv/Fm	Clover Fv/Fm
C	1	0.87 ± 0.01	0.88 ± 0.01
	2	0.87 ± 0.01	0.86 ± 0.01
	3	0.87 ± 0.02	0.87 ± 0.02
	4	0.85 ± 0.02	0.87 ± 0.01
	5	0.87 ± 0.02	0.86 ± 0.01
D	1	0.86 ± 0.01	0.86 ± 0.01
	2	0.86 ± 0.01	0.87 ± 0.02
	3	0.86 ± 0.01	0.86 ± 0.02
	4	0.85 ± 0.01	0.85 ± 0.03
	5	0.86 ± 0.01	0.85 ± 0.02
H	1	0.85 ± 0.02	0.83 ± 0.03
	2	0.85 ± 0.02	0.80 ± 0.05
	3	0.86 ± 0.01	0.80 ± 0.03
	4	0.85 ± 0.01	0.77 ± 0.08
	5	0.85 ± 0.02	0.77 ± 0.04
D+H	1	0.88 ± 0.02	0.82 ± 0.03
	2	0.86 ± 0.01	0.76 ± 0.08
	3	0.86 ± 0.01	0.75 ± 0.03
	4	0.82 ± 0.03	0.79 ± 0.02
	5	0.47 ± 0.15	ND

Chapter 6
Combined Abiotic Stress in Legumes

Santiago Signorelli, Esteban Casaretto, Jorge Monza and Omar Borsani

6.1 Legume Family: Agronomic Relevance

A major source of protein in the human diet is of animal origin. The production of beef and mutton is based on natural pastures or supplementation based on grains (feedlot). Sown pastures can be monospecific or may be ultrasimple, simple or complex of different species of the same botanical family or a family of different botanical blends. Within the latter group, are mixtures of grasses and legumes.

From the point of view of human and animal consumption, legumes belonging to the subfamily Papilonideae are relevant. This includes seeds and forage legumes such as peanut, beans, chickpea, broad beans, lentils, soybean, among others. Some species of the genus *Medicago, Lotus* and *Adesmia* can be used as forage or green manure, thus enhancing the contents of nitrogen in the soils.

Forage legumes have been widely spread in the world due to the great agronomic importance that they possess. The species of this plant family are an invaluable component of pastures, mainly due to their ability to fix atmospheric nitrogen through symbiotic association with several bacteria collectively called *rhizobia*. Second, legumes have a high nutritional value, especially proteins and minerals (Ca^{+2} and Mg^{+2}), which makes them essential for the production of forage. Legume crops also play a critical role as main protein sources in vegetarian diets. Tolerance

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to environmental abiotic stress is one of the ways to improve the productivity of legumes and aid in harnessing their potential nutritional value. Identification of biochemical and physiological characters which contribute to improve the yield in legumes under limiting conditions is a main objective of plant breeders for agricultural and cattle-rearing regions. Thus, this chapter intends to provide an understanding of the mechanisms involved in the combined stress-tolerance responses in legumes.

6.2 Environmental Stresses Induce Varied Plant Responses

Plants are frequently subjected to stress—environmental condition that adversely affects the growth, development and productivity thereof. Biotic stress can be imposed by organisms such as viruses, bacteria and fungi, while abiotic stress can be due to an excess or deficit in some environmental factor. Among the environmental conditions that cause damage are excess water, water deficit, soil salinity, extreme temperatures, insufficient mineral nutrients in the soil and high- or low-light radiation (Bohnert and Sheveleva 1998; Bray et al. 2000).

Resistance or susceptibility to stress depends on the species, genotype and stage of development of the plant. Resistance mechanisms can be grouped into two categories—those that prevent exposure to stress and the other that results in tolerance. Certain morphological features such as sunken stomata and deep roots are examples of resistance mechanism that can prevent stress. However, other mechanisms of resistance are achieved by acclimation, i.e. the maintenance of internal homeostasis of the various organelles in response to changing environmental factors (Bray et al. 2000).

Plants acclimate to manage the different types of stress triggering a wide range of responses from the perception of stress at the cellular level, leading to the activation of a very large number of genes. Key components of the stress response are the stimulus itself, transducers, signal molecules, transcription regulators, responsive genes that trigger morphological, biochemical and physiological adaptation involved in this situation. In turn, the duration and severity with which stress is imposed determine how the plant will respond (Pastori and Foyer 2002; Bray et al. 2000).

Unlike resistance to biotic factors, resistance to water stress and other abiotic factors, despite being clearly genetic, is not a result of the action of a specific gene (Zhu et al. 1997). The ability of plants to withstand water stress is a multigenic trait and biochemical pathways responsible for products or processes that improve the overall strength can act additively, and also synergistically (Bohnert et al. 1995).

It is reported that several genes responsive to water stress not only perform their functions protecting cells by producing metabolically important proteins under water deficit but also in the regulation of genes involved in signal transduction in response to stress. Thus, these gene products are classified into two groups: The first group includes proteins that are involved in stress tolerance such as channel

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proteins involved in the movement of water across membranes, enzymes necessary for the biosynthesis of osmolytes, proteases and macromolecules that can protect membranes, among others. The second group includes factors involved in the regulation of signal transduction and gene expression, such as protein kinases, transcription factors and 14-3-3 proteins, among others (Bray 1997; Shinozaki and Yamaguchi-Shinozaki 1997).

Higher temperatures primarily affect photosynthesis, in particular CO_2 assimilation because Rubisco activation is inhibited. Plants exposed to excessive temperatures have specific metabolic cellular response characterized by low protein synthesis, and induction of the synthesis of heat shock proteins (HSPs). In addition to altering the pattern of gene expression, the high temperature can damage cellular structures such as organelles and cytoskeleton (Bray et al. 2000; Tang et al. 2007).

Water stress and high temperatures interact strongly with each other and have opposite effects on photosynthesis. For example, in response to high temperature, plants open their stomata to cool their leaves by transpiration, but if there is also water deficit condition, plants would not be able to open the stomata and hence leaf temperature will increase (Rizhsky et al. 2002). While both types of stress have been extensively studied individually, few studies (Lu and Zhang 1999; Rizhsky et al. 2002; Rizhsky et al. 2004) focused on impacts of combined heat and water stress—a common situation prevailing under field conditions. It is possible that combination of these stress factors can alter the metabolism of the plant differently, compared to when a single stress is imposed (Xu and Zhou 2006).

6.2.1 Plants Response to Water Stress

Water deficit is one of the most widespread environmental factor stresses that occurs when the transpiration rate exceeds the absorption of water from the root system. Water deficit at the cellular level may result in an increase of solute concentration, changes in cell volume, disruption of water potential gradient, turgor loss, loss of membrane integrity and protein denaturation. The ability of the plant to respond to water deficit and survive depends on mechanisms that involve the integration of cellular responses throughout the plant (Bray et al. 2000).

Water deficit is a common plant environmental stress that dramatically limits growth and development. Water stress can trigger a significant decrease in crop productivity and quality, especially evident in grain and forage legumes. *Lotus japonicus* is a well-established model legume closely related to forage legumes such as *Lotus corniculatus, Lotus tenuis* and *Lotus uliginosus* (Choi et al. 2004; Díaz et al. 2005a). Alfalfa is a legume species with great plasticity that can succeed in semiarid, subhumid and humid regions and for that reason is called the "queen of forage legumes". However, it requires well-aerated and deep soils and is morphologically and physiologically adapted to withstand prolonged water deficiencies. In marked contrast to their drought-tolerant nature, these plants are very sensitive to a lack of oxygen that is common in flooding soils.

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Legumes are typically subjected to a variety of different environmental stresses such as water stress. At the cellular level, this stress induces overproduction of reactive oxygen species (ROS; Fig. 6.1), such as hydrogen peroxide (H_2O_2), superoxide radical ($O_2^{\bullet -}$) and hydroxyl radical ($^{\bullet}OH$), which are responsible for oxidative damage associated with stress (Dat et al. 2000). Plants respond to stress using different enzymatic and non-enzymatic antioxidant systems. Oxidative stress responses may involve increased activity of superoxide dismutase (SOD), catalase (CAT) and ascorbate–glutathione cycle activities such as glutathione reductase (GR) or ascorbate peroxidase (APX), which can confer greater tolerance against a specific environmental stress (Sade et al. 2011). Increased levels of non-enzymatic soluble antioxidants including glutathione (GSH), ascorbic acid and tocopherols are also produced in response to water stress-induced oxidative stress (Feng et al. 2004). Plant antioxidant defence systems normally provide adequate protection against ROS damage under optimal growth conditions. The generation of higher levels of ROS may overcome the defence provided by these systems and result in oxidative stress (Mittler 2002; Noctor and Foyer 1998; Valderrama et al. 2006). Cellular damage caused by oxidative stress includes lipid peroxidation, which increases in various tissues during water stress and is also a common marker of oxidative stress (Sade et al. 2011).

In response to water deficit, plant cells also accumulate low-molecular-mass compounds termed compatible solutes, mainly proline, glycine betaine, sugars and polyols, in the cytoplasm to control the ionic balance in the vacuoles (Parida and Das 2005). Among these solutes, proline has been associated with different

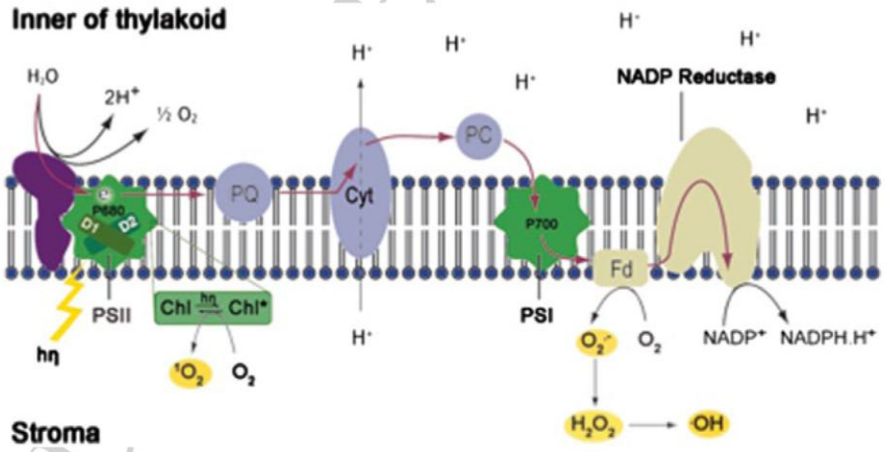

Fig. 6.1 ROS production in the chloroplast. *Chl* chlorophyll, *Chl** excited chlorophyll. *PSI* photosystem I, *PSII* photosystem II. *Cyt* cytochrome, *PQ* plastoquinone, *PC* plastocyanin. Superoxide ($O_2^{\bullet -}$) can be produced by electron transfer to oxygen. Hydrogen peroxide (H_2O_2) is produced from superoxide by spontaneous dismutation or SOD activity. Hydroxyl radicals ($^{\bullet}OH$) are produced from hydrogen peroxide by homolysis or Fenton reaction in the presence of Fe^{3+}. Singlet oxygen is generated from oxygen by energy transfer from excited chlorophylls

functions, such as being a free radical scavenger, a cell redox balancer, a cytosolic pH buffer and a stabilizer for subcellular structures, especially during osmotic and salt stresses (Szabados and Savouré 2010).

During drought establishment, plants exhibit a decrease in stomatal conductance with the consequent decrease in CO_2 assimilation. Stomatal closure has been considered as the main reason for the inhibition of photosynthesis under drought. However, it was demonstrated that limiting stomatal water losses is not so important to maintain photosynthetic activity. For example, it has been observed in leaves of various species, reductions in photosynthesis occur without apparent effects on stomatal conductance (Teskey et al. 1986; Hutmacher and Krieg 1983), suggesting that factors independent of stomatal behaviour impact photosynthesis in plants subjected to drought.

The use of split root system has helped in gaining knowledge about the impact of drought on the process of nodulation in legumes (Larrianzar et al. 2014). Nodule number is mainly regulated at the systemic level through a signal which is produced by nodule/root tissue, translocated to the shoot and transmitted back to the root system. This process involves shoot Leu-rich repeat receptor-like kinases. In contrast, local and systemic mechanisms regulate nitrogenase activity in nodules (Esfahani et al. 2014). Under drought and heavy metal stress, the regulation is mostly local, whereas the application of exogenous nitrogen seems to exert a regulation of nitrogen fixation both at the local and systemic levels (Marino et al. 2007).

6.2.2 Response of Plants to Heat Stress

High temperature at early sowing resulted in poor crop establishment due to failure of seed germination, emergence and reduced vigour (Khalaffalla 1985; Weaich et al. 1996). In such situations, avoidance mechanisms, such as transpiration, leaf rolling, hairiness or wax layers, may play a role in dissipating the heat load. However, in general, transpiration is the most important heat-dissipating system through latent heat loss (Kramer 1983).

Plants exposed to high temperatures, at least 5 °C above their optimal growing conditions, exhibit cellular and metabolic responses required for the plants to survive under this condition (Guy 1999). These effects include changes in the organization of organelles, cytoskeletal reorganization and membrane functions, accompanied by a decrease in the synthesis of some proteins and overexpression of HSPs, the production of phytohormones such as abscisic acid (ABA) and antioxidants and other protective molecules (Bita and Gerats 2013; Maestri et al. 2002; Bray et al. 2000). Under heat stress, about 5 % of plant transcripts (~ 1500 genes) are up regulated, twofold or more (Rizhsky et al. 2004; Larkindale and Vierling 2008; Finka et al. 2011). A significant fraction of these transcripts encode heat-induced chaperones. For example, 88 out of 1780 in *Arabidopsis thaliana,* and 117 out of 1509 in wheat, are associated with HSP-based protection mechanism (Liu et al. 2008; Ginzberg et al. 2009; Bokszczanin and Fragkostefanakis 2013).

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There are many transcripts-encoding proteins involved in calcium signalling; protein phosphorylation; phytohormone signalling; sugar and lipid signalling and metabolism; RNA metabolism; translation, primary and secondary metabolisms; transcription regulation and responses to different biotic and abiotic stresses (Mittler et al. 2012; Huve et al. 2011). Changes in ambient temperature are sensed by plant sensors positioned in various cellular compartments. The increased fluidity of the membrane leads to activation of lipid-based signalling cascades and to an increased Ca^{2+} influx. Signalling by these routes leads to the production of osmolytes and antioxidants as a response to heat stress. This stress also brings about changes in respiration and photosynthesis and thus leads to a shortened life cycle and diminished plant productivity (Barnabás et al. 2008).

The early effects of heat stress comprise of structural alterations in chloroplast–protein complexes and reduced activity of enzymes (Ahmad et al. 2010). The photochemical modifications in the carbon flux of the chloroplast stroma and those of the thylakoid membrane system are considered the primary sites of heat injury (Wise et al. 2004), as photosynthesis and the enzymes of the Calvin–Benson cycle, including ribulose 1,5-bisphosphate carboxylase (Rubisco) and Rubisco activase are very sensitive to low increases of temperature, and it is suggested to be one of the primary determinants of heat-dependent reduction in photosynthesis (Maestri et al. 2002; Morales et al. 2003). Heat inactivation of Rubisco is reversible (Salvucci and Crafts-Brandner 2004; Kim and Portis 2005). However, moderate heat stress has been shown to alter the thylakoid permeability and electron transport (Schrader et al. 2007; Zhang and Sharkey 2009), and this inhibition of electron transport is associated with enhanced membrane permeability, disorganization of photosystem II (PSII) and antenna tertiary structure, and disruption of the water splitting and oxygen evolving system (Huve et al. 2011). Other specific responses of heat stress on photosynthetic membranes include the swelling of grana stacks and an aberrant stacking. Such structural changes are accompanied by ion leakage from leaf cells exposed to heat and changes in energy allocation to the photosystems (Wahid and Shabbir 2005; Allakhverdiev et al. 2008). The maintenance of cellular membrane function under heat stress is thus essential for sustained photosynthetic and respiratory performance (Chen et al. 2010). The detrimental effects of heat on chlorophyll and the photosynthetic apparatus are also associated with the production of ROS (Guo et al. 2007). By increasing chlorophyllase activity and decreasing the amount of photosynthetic pigments, heat stress ultimately reduces the plant photosynthetic and respiratory activity (Sharkey and Zhang 2010).

Homeostasis, in general, including biosynthesis and compartmentalization of metabolites, is disturbed in high-temperature-challenged plant tissues (Maestri et al. 2002). Among the primary metabolites, accumulating in response to heat stress are proline, glycine betaine or soluble sugars (Wahid 2007).

Heat stress results in the misfolding of newly synthesized proteins and the denaturation of existing proteins. Protein thermostability is provided in part by chaperones (Ellis 1990). In this sense, the exacerbation of combined heat and other stress could be due to the loss of function of some enzymes that are overexpressed in response to other stress.

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6.3 Effect of Water Stress–Heat Stress Combination on Different Plant Processes

L. corniculatus and *Trifolium pratense* are legumes used in agriculture as a forage source. These species are both perennial herbaceous plants used in temperate grassland and can be nodulated by *rhizobia*. Nevertheless, lotus is better suited to soils with water restriction and has a superior tolerance to water stress (Peterson et al. 1992). In the field, mainly during summer, these plants are commonly exposed to environmental stresses such as water stress and high temperatures, which in fact are considered to be the most important environmental factors limiting plant growth and development (Berry and Bjorkman 1980; Yordanov et al. 1986; Sinsawat et al. 2004).

6.3.1 Proline Accumulation

The accumulation of proline is known to be a good indicator of water stress in *L. corniculatus* (Díaz et al. 2005b). However, the responses to combination of stresses are not a mere additive effect of the single stresses. For example, some plants that tend to accumulate proline in water stress conditions replace it with sucrose as the major osmoprotectant when subjected to a combination of water stress and heat stress (Rizhsky et al. 2004). In *L. corniculatus* water stress and heat individually produce proline accumulation, but concomitant imposition of both stresses produced a higher accumulation of proline. In contrast, *Trifolium Pratense*-accumulated proline in water stress conditions but not under heat stress and the imposition of the combined stress produced only a slight increase in proline concentration compared to unstressed plants (Signorelli et al. 2013). Thereby, for *L. corniculatus*, proline accumulation is a parameter that can be used as a stress marker to assess water stress and heat stress conditions, as well as the combination of both. However, proline accumulation in legumes cannot always be considered a good indicator of stress condition when two or more stresses are present. It is also known that proline accumulation under heat stress decreases the thermotolerance of the plant, probably because of an enhancement in the production of ROS via the Pro/P5C cycle (Lv et al. 2011). In *T. pratense,* it was suggested that blocking proline accumulation might be a strategy to avoid self-toxicity during heat stress (Signorelli et al. 2013). This hypothesis correlated with the lipid peroxidation estimated by thiobarbituric reactive substances (TBARS), as *T. pratense* did not show an increase in lipid peroxidation under heat conditions. Moreover, *T. pratense* has a lower lipid peroxidation content than *L. corniculatus* when water stress and heat stress are combined—a treatment in which *L. corniculatus* accumulates the highest levels of proline.

On the other hand, it has been demonstrated that proline can act as an osmolyte under severe dehydration (Verslues and Sharp 1999). The non-accumulation of proline and the greater leaf area of *T. pratense* are important disadvantages of

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this species compared to *L. corniculatus* when water loss must be prevented. In a comparative analysis of *L. corniculatus* and *T. pratense* subjected to water stress and heat, it was observed that *T. pratense* did not survive 5 days of combined stress, while lotus was still alive (Signorelli et al. 2013c). In concordance, higher dry-matter yield was observed in lotus compared to *T. pratense* under field conditions subjected to summer water stress (Peterson et al. 1992).

6.3.2 Oxidative Stress

Most stresses induce ROS and alter the antioxidant–enzymatic response (Mahalingam and Fedoroff 2003). However, little is known about how two or more stresses affect the ROS production and the antioxidant response. In the model, legume *L. japonicus* (Sainz et al. 2010) and the forage legumes *L. corniculatus* and *T. pratense* was investigated how water stress and heat modify the antioxidant response and oxidative damage (Signorelli et al. 2013).

SOD is the main enzymatic system responsible for cell detoxification and is well documented in several plant species to increase in response to water deficit and heat stress (Alscher et al. 2002). In *L. corniculatus*, the activity of Mn-SOD and Fe-SOD increased as a consequence of water stress and combined stress (Fig. 6.2), but it did not change under heat stress (Fig. 6.2). In the related model specie *L. japonicus,* Cu/Zn-SOD immunodetection and the isoenzyme-specific activity assays confirmed that high-temperature treatment provoked a reduction in the Cu/Zn-SOD protein content and activity. This is consistent with a failure to convert $O^{2.-}$ to H_2O_2 in the combined heat–drought condition. Additionally, in spite of the decreased Cu/ZnSOD in the high-temperature treatment, the accumulation of $O^{2.-}$ remains low,

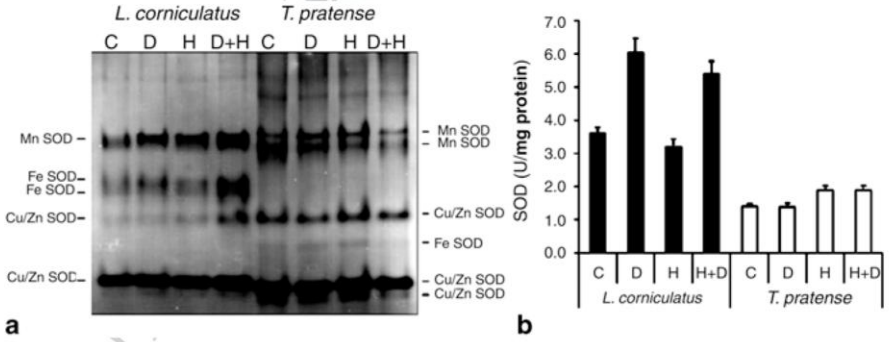

Fig 6.2 SOD activity under drought and combined heat and drought stress. **a** SOD isoforms profile. *C* control; *D* drought; *H* heat at 42 °C; D + H drought + heat at 42 °C. 40 and 200 mg of protein were loaded in *L. corniculatus* and *T. pratense,* respectively. The gel is the most representative of three replicates of native gels. **b** Total in vitro SOD activity. *C* control; *D* drought; *H* heat at 42 °C; D + H drought + heat at 42 °C. One unit of SOD was defined as the amount of enzyme that inhibits the rate of cytochrome c reduction by 50 %. *Bars* indicate the relative standard deviation. (Figure modified from Signorelli et al. 2013)

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and this is likely because high temperature does not induce accumulation of this ROS (Sainz et al. 2010).

In *T. pratense,* however, no changes were observed in the activities of any SOD isoforms. The results of the quantitative enzyme activity assay demonstrated that total SOD activity is 2.6-fold greater in *L. corniculatus* than in *T. pratense,* and it is affected by the stress treatments. Heat did not modify the SOD activity in *L. corniculatus,* but the combination with water stress led to same level activity observed under water stress (Fig. 6.2). *T. pratense* showed a slight increase in the SOD activity by heat stress and combined stress (Fig. 6.2). In this case, for both legumes the response of SOD activity in the combined stress was the addition of responses in the individuals' stresses. It could be concluded that if one of the stresses that produce the induction of SOD activity is present, the induction of SOD activity will be warranted in the combined stress. In *L. japonicus,* heat stress led to a decrease on Cu/Zn-SOD contents, which also was observed under a combination of heat and water deficit (Sainz et al. 2010).

In *L. corniculatus,* CAT activity only increases during the combination of water stress and heat. However, in *T. pratense*, CAT enzyme activity increased with reference to control in response to water deficit, heat stress and combined stress, although no differences were observed among these stresses. In *T. pratense,* it was observed that any stress was able to induce CAT activity and the combination of both stresses did not lead to an additive effect on the enzyme activity. For *L. corniculatus,* it seems that any individual stress is not sufficient to induce CAT activity; however, the combination of stresses led to the induction of CAT, suggesting that more than one signal is required to induce this enzyme. In *L. japonicus,* the combination of heat and water deficit led to an increase in CAT activity, that was much higher than the activity observed when the stressors were imposed individually (Sainz et al. 2010).

Interestingly, the APX activity in *L. corniculatus* was inhibited by water stress condition, while in *T. pratense,* this activity was inhibited only in the combined stress treatment. This enzyme is inactivated by nitration (Begara-Morales et al. 2014), which is reported to occur under several abiotic stresses (Corpas et al. 2013). For example, for *L. japonicus,* a closely related species, it was observed that water deficit induces a nitro-oxidative stress that was also reducing APX activity (Signorelli et al. 2013c). We speculate that the different stressful situations are also inducing nitro-oxidative stress in these plants, and this could explain the decay in enzyme activity.

Both *L. corniculatus* and *T. pratense* leaves showed $O_2^{\bullet-}$ accumulation only in the water deficit–heat stress combination, as was previously observed in the model legume *L. japonicus* (Sainz et al. 2010). The higher SOD activity in water stress conditions with respect to controls, would allow this species to deal with the $O_2^{\bullet-}$ induced mainly by water stress. However, the increase of Mn-SOD and Fe-SOD isoform activity by water stress was lost under high-temperature conditions, resulting in an increase of $O_2^{\bullet-}$ in the combined treatment. In *L. japonicus,* similar results were obtained with Cu/Zn-SOD, showing that deleterious effects of heat stress on SOD activity might be a general response for this legume genus (Sainz et al. 2010).

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The differences detected between both species are mainly explained by changes in the Cu/Zn- SOD isoforms. In *T. pratense,* H_2O_2 accumulation showed the same pattern; however, in *L. corniculatus,* the highest accumulation of ROS was observed under water deficit. These results clearly demonstrate that combination of stress situations cannot be always considered the additive responses of individual stresses.

L. corniculatus showed an increase in TBARS content as a consequence of water deficit, heat stress and a combination of these. But *T. pratense* did not produce any increase in TBARS content under heat stress. The absence of proline accumulation in *T. pratense* may be an advantage under heat stress by avoiding the Pro/P5CS cycle which, as previously mentioned, could result in higher ROS production via the Pro/P5CS cycle (Lv et al. 2011). However, proline accumulation might be critical under combined stress because the osmolyte function seems to be important when water stress is established.

6.3.3 Photosynthesis

Water stress and heat combination affects the rate of photosynthesis due to an increase in photoinhibition, a process that can be enhanced when more types of abiotic stress coexist (Takahashi and Murata 2008). Under stress conditions, the possibility of overexcitation of PSII increases. This can cause a decline in the photosynthetic rate as the process of photoinhibition increases due to the necessity to dissipate, through nonradiative processes, the excess of absorbed energy (Takahashi and Murata 2008; Baker 2008). Because the capacity of photoprotection is limited, certain conditions can lead to damage and loss of active PSII reaction centres. Under severely high temperatures, in combination with water stress, the photosynthetic apparatus is the primary site of damage. On the contrary, photosystem I is more resistant to heat than PSII (Sayed et al. 1989; Hu et al. 2004; Havaux 1993). Once photoinhibition is established, the PSII reaction centre is simultaneously repaired via removal, synthesis and replacement of degraded D1 protein (Ohad et al. 1984; Kyle and Ohad 1986), a protein of reaction centre of PSII (Fig. 6.1). The observed photoinhibitory damage is the net result of a balance between photodamage and the repair process (Samuelsson et al. 1985; Lidholm et al. 1987; Shyam and Sane 1989). Several studies have reported a good correlation between changes in chlorophyll fluorescence parameters in response to environmental stresses, such as heat, chilling, freezing and salinity (Bonnecarrére et al. 2011; Smillie and Hetherington 1983; Yamada et al. 1996; Hakam et al. 2000). Others authors have linked the decrease in the maximum quantum yield of PSII (F_V/F_M) to the physical dissociation of the PSII reaction centres that lead to photoinhibition, and this assay was used to identify tolerant wheat cultivars (Abdullah et al. 2011).

In *L. corniculatus,* no changes of the maximum quantum efficiency, evaluated as F_V/F_M, were observed in any treatment until the 5th day, when the combined treatment showed a significant decrease in the F_V/F_M parameter. In contrast, in *T.*

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pratense, this fluorescence parameter slightly decreased from the 1st day in the heat and combined treatment, but no changes were observed under water stress conditions.

L. corniculatus showed a slight decrease in the amount of D1 protein after water stress treatment. However, there was no decrease in the protein content when the control and heat conditions were compared. The D1-complex profile of *T. pratense* was also analysed, and the western blot showed a very different result when compared with the *L. corniculatus* profile. The total D1 protein content in *T. pratense* did not change in any treatment, but a difference was found in the ratio between the free protein and the complex form. In the treatments where heat was involved, an increase in free D1 protein together with a decrease in the D1-complex form was evident, but it should be considered that this result might be a consequence of the high hydrophobicity of these complexes, which makes their isolation difficult. Regarding D2, in *L. corniculatus,* the results were similar to those observed with D1; namely, when water stress was present in the treatments, a reduction in the amount of D2 protein was observed. Surprisingly, in the combined treatment, the D2 protein was not detected. In contrast, in *T. pratense*, no significant changes were observed in D2 protein levels.

The chlorophyll fluorescence parameter that was evaluated showed that *L. corniculatus* had a significant decrease in the maximum quantum efficiency at the end of the combined treatment. The low F_V to F_M ratio indicated photoinhibition, a process that can act as determinant of plant performance during a stress condition (Abdullah et al. 2011). In *T. pratense,* only a small decrease in the F_V/F_M values was observed from the 1st day and in the treatments involving heat stress.

Analysis of PSII proteins in the two legumes with contrasting water stress responses shows an effect that is stress- and species-specific. The D1 and D2 subunit content is decreased in *L. corniculatus* in both treatments involving water stress, showing certain adaptability in response to water stress. Interestingly, the decrease in the D2 levels was pronounced in the combined treatment, and this is well correlated with the decrease in the maximum quantum efficiency, suggesting the presence of a disassembling process. The D2 subunit is of particular interest because it represents the initial point for the assembly of the PSII as a whole (de Vitry et al. 1989; Komenda et al. 2004; Minai et al. 2006). The expression of the gene that encodes the D2 subunit of the PSII reaction centre is regulated post-transcriptionally by an RNA-binding protein (Schwarz et al. 2007). Modifications induced by the stress in this post-transcriptional regulation could be a possible explanation for the absence of D2 in *L. corniculatus* subjected to the combined stress treatment.

In *T. pratense,* the total content of D1 and D2 did not change, but we observed an increase in the free form of D1 in treatments involving heat. One possible explanation is that the turnover of D1 is taking place in the heat treatments, and this is evident based on the increase of free D1 together with a reduction of the D1–D2 complex, as well as a decrease in the F_V/F_M values.

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6.4 Waterlogging and Salinity: A Combined Stress in Legumes

Salt stress is certainly one of the most serious environmental factors limiting the productivity of crop plants (Ashraf and O'Leary1999). Salinity reduces the ability of plants to take up water, causing rapid reductions in growth rate, along with an array of metabolic changes identical to those caused by water stress (Munns 2002).

High salt concentration in the external solution of plant cells produces several deleterious consequences. First, salt stress causes an ionic imbalance (Niu et al. 1995). The homeostasis of not only Na^+ and Cl^- but also K^+ and Ca^{+2} ions is disturbed (Rodriguez-Navarro 2000; Hasegawa et al. 2000; Serrano et al. 1999). As a result, plant survival and growth will depend on adaptations that re-establish ionic homeostasis, thereby reducing the duration of cellular exposure to ionic imbalance. Second, high concentrations of salt impose a hyperosmotic shock by decreasing water and causing loss of cell turgor. This negative effect in the plant cell is thought to be similar to the effects caused by drought. Third, reduction of chloroplast stromal volume and generation of ROS, in salt-induced water stress, are also thought to play important roles in inhibiting photosynthesis (Price and Hendry 1991). On the molecular level, these responses are manifested as changes in the pattern of gene expression (Maggio et al. 2002).

The process of salinization results from the interaction between climate, geomorphology, hydrology, land use and surface water properties and dynamics of the salts. Regions with salinity are frequently associated with geographical localization with inundation events; thus it is not infrequent that salt and flood stress occurs simultaneously.

Salinity and waterlogging interact adversely to reduce production of crops and pastures, as very few species used in agriculture can tolerate the combination of both stresses (Barrett-Lennard 2003). Moreover, annual pasture legumes are particularly sensitive to combined salinity and waterlogging (Bennett et al. 2009).

One of the most important consequences of energy limitation under anoxia is altered redox state of the cell. Under low oxygen pressure conditions, the intermediate electron carriers in electron transport chain become reduced, affecting redox-active metabolic reactions. Therefore, for maintaining redox homeostasis cells need to regulate NADH to NAD ratio under flooding (Chirkova et al. 1992). Saturated electron transport components, the highly reduced intracellular environment and low-energy supply are the factors favourable for ROS generation. The consequences of ROS formation depend on the intensity of the stress as well as on the physicochemical conditions in the cell (i.e. antioxidant status, redox state and pH). As was mentioned for other stresses, ROS accumulation may cause damage to different cell structures and biomolecules. H_2O_2 production during O_2 deprivation was observed in the plant cells (Blokhina et al. 2001), and its degradation was found to play an important role in waterlogging tolerance in non-legume plants (Lin et al. 2004).

A trait that is essential for root survival during water logging or flooding is the development of aerenchyma (Armstrong 1979). Aerenchymas are cortical airspaces

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that provide a low-resistance internal pathway for the movement of O_2 from the shoots to the roots, where it is consumed in respiration and may also reoxidize the rhizosphere (Armstrong 1970; Armstrong 1971, 1979). In legumes, aerenchyma may also be important for supplying O_2 and N_2 to root nodules (Walker et al. 1983; James et al. 1992; Zook et al. 1986; Pugh et al. 1995). Tolerance of *Melilotus siculus* to waterlogging is associated with the production of a highly porous phellem, a type of secondary aerenchyma, on taproots and upper lateral roots (Verboven et al. 2011).

Studies with plant species sensitive or tolerant to flooding–salt stress combination have shown that the rate of transport of Na^+ and Cl^- to the shoot is critical to define the response. The ions transport rate increases significantly under combined stress in comparison with salinity alone (Barrett-Lennard 2003). For more tolerant species, there is only small or even no increase in shoot Na^+ and Cl^- in response to combined salinity and waterlogging (Colmer and Flowers 2008), presumably due to better root aeration. Moreover, in perennial legumes such as *Trifolium repens L.* (Rogers and West 1993) and *Liolaemus tenuis* (Teakle et al. 2007), high root porosity was associated with better shoot ion regulation under combined salinity and waterlogging. Comparisons of annual pasture legumes in growth, ion regulation and root porosity demonstrate that *M. siculus* has exceptional tolerance to combinations of salinity and waterlogging (Teakle et al. 2012). Enhanced root aeration would avoid energy deficits that could impair ion transport processes in roots, which determines delivery of Na^+ and Cl^- to shoots via the xylem (Barrett-Lennard 2003; Teakle et al. 2007; Colmer and Flowers 2008). Thus, traits of importance for tolerance to combined salinity and waterlogging are likely to include high root porosity, leading to decreased shoot Na^+ and Cl^- concentrations.

AQ3

6.5 Metabolic Changes in Responses to Stress Combination

It is well known that the effect of a combination of different stresses on plants can be quite different from those generated when plants are subjected to individual types of stress (Rizhsky et al. 2002). Table 6.1 represents a summary of how the combination of different stresses affects some parameters in legumes.

With reference to antioxidant responses, different patterns are observed when more than one stress is imposed. However, it seems that in most cases the addition of other stress did not alter the response. It implies that the signal molecules that induce the expression of antioxidant enzymes probably are the same in different stresses and so the imposition of both stresses is redundant. In other cases, the effect of simultaneous stresses produces deleterious effects. For example, for APX and CAT, one stress produces the induction of the activity (or at least a normal level of activity), but the imposition of two stresses could produce a more nitrosative condition in the cell leading to the nitration of the enzyme, which is known to decrease the activity of these enzymes.

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Table 6.1 Effects of stress combination on main parameters studied in legumes

		Response			
	Evaluated parameter	Negative correlation	Unchanged	Additive response	Synergistic effect
Antioxidant enzymes	APX				
	SOD				
	CAT				
	GR				
	POX				
Oxidative stress	H_2O_2				
	TBARS				
	Electrolyte leakage				
	$O_2^{\circ-}$				
Photosynthetic activity	F_V/F_M				
	CP47				
	D1				
	D2				
Metabolites	Proline				
	Ascorbic acid				

Darker shading indicates that the particular response is supported by more evidence. Data obtained from following legume species under various combined stresses: *L. corniculatus, T. pratense* subjected to combined drought and heat (Signorelli et al. 2013a), *L. japonicus* subjected to combined drought and heat (Sainz et al. 2010), *Vigna unguiculata* subjected to combined CO_2, UV-B radiation and temperature stress (Singh et al. 2010), *Vigna radiata* (Siddiqui 2013) and *Phaseolus vulgaris* subjected to combined zinc and high irradiance stress (Michael and Krishnaswamy 2011).

APX ascorbate peroxidase, *SOD* superoxide dismutase, *CAT* catalase, *GR* glutathione reductase, H_2O_2 hydrogen peroxide, *TBARS* thiobarbituric reactive substances, $O_2^{\bullet-}$ superoxide radical, F_v/F_M photosystem II

Among the oxidative stress markers, synergistic effect was the most commonly observed response. Most stresses are accompanied by an increment of ROS production, and the source of ROS is different for different stresses (Mahalingam and Fedoroff 2003; Wrzaczek et al. 2013). Thus, when more than one stress is present, it induces ROS from different organelles, and hence the total ROS tends to be higher in combined stress scenarios. Less commonly, a negative correlation or an unchanged response is observed. In one case of negative correlation observed for H_2O_2, it was suggested that the reduction in SOD activity in combined stress as opposed to in single stress was responsible for the lower H_2O_2 in the former. In the other case, induction of CAT activity only in the combination of stress was suggested to be the cause of lower H_2O_2 levels.

Photosynthetic activity does not show a defined pattern, maybe due to lack of information. Even with the limited data, it can be seen that in all the cases examined, D1 was unchanged by the imposition of combined stresses. D2 protein had a synergistic effect in combined stress. It is important to point out that in drought and heat stress were considered in these studies, and some of these responses were observed in *T. pratense* and in two related species such as *L. japonicus* and *L. corniculatus*. Other species should be evaluated to see the conservation in the response of D2, which is suggested to disassemble to induce inhibition of photosystem activity, and protect cells from oxidative damage caused by its own activity.

6.6 Forage Legumes Field Productivity and Combined Environmental Stress

Legumes have a high level of productive diversification and flexible utilization. The same species can be usefully exploited for different purposes such as soil protection from erosion; green manure crop; mulching; cover crop in vineyards, orchards and firebreak lines; high quality honey production; landscape enhancement and medicinal use. Consequently, forage legumes were adapted to a wide range of soil types, climatic conditions and management systems (Sánchez-Díaz 2001).

Legumes, as many other crops, have been bred to maximize productivity (forage or grain). But this productivity is always affected by adverse environmental factors. Perennial forage legumes are a good model to analyse the responses of adaptability of plants under field conditions. This is because during the whole plant growth and development cycle, plants are subjected to various types of abiotic stresses, both singly and in combinations.

Low temperatures and periods of water saturation in soils are common during the winters in many regions and in the other side periods of low water regime combined with high temperatures are common during summers. To these we must add other combinations of stresses such as periods of high radiation or toxic ions (Na^+ or heavy metals) produced by changes in the physicochemical conditions of the soils.

Further, abiotic stress can affect the legume plants at different developmental stages. So legumes growing under field conditions must have adaptation process triggered by stress in seedling, vegetative or reproductive stages. For example, for seedling emergence, the optimal conditions in the field are established at the end of winter (Fig. 6.3).

Legumes are adapted to different environmental conditions by setting the developmental stages, such as reseedling capacity that is an important characteristic for the perpetuation of *L. corniculatus*. Yield of *L. corniculatus* during 3 years with seed set and without seed set, reveal the importance in reseedling (Fig. 6.4, Ayala and Carámbula 2009).

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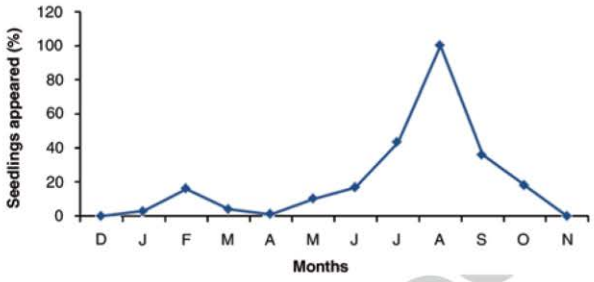

Fig. 6.3 Seedlings emergence of *L. tenuis* during a typical of temperate zones from south hemisphere (Ayala and Carámbula 2009)

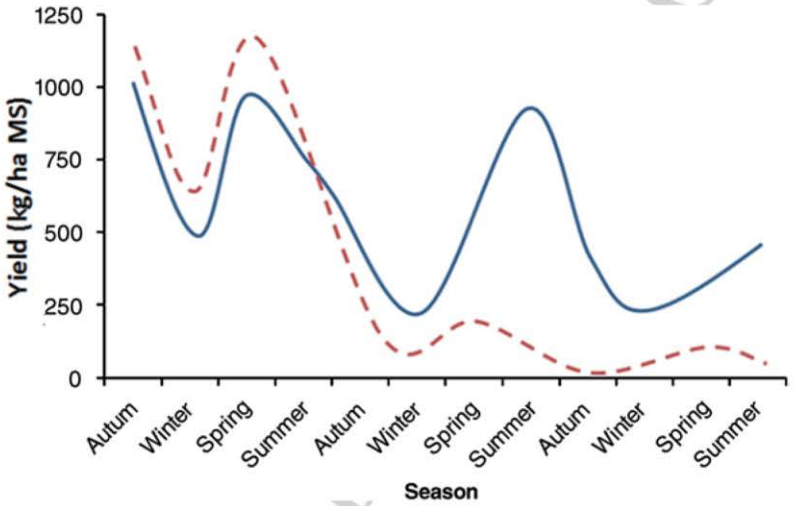

Fig. 6.4 Seasonal dry matter production of *L. corniculatus* under two-seed set management. With seed set (*solid line*) and without seed set (*dashed line*). (Ayala and Carámbula 2009)

538 This suggests that the tolerance in reproductive stages should be accompanied
539 by physiological responses to deal with water restriction and high temperatures.
540 Another key physiological mechanism in the survival of legumes is their ability to
541 mobilize carbohydrates to storage tissues that can be located on the crown, root or
542 rhizome (Castillo et al. 2012). However, it is critical that the photosynthetic activ-
543 ity remains active during the stress period to achieve significant accumulation of
544 sugars allowing regrowth of the shoot after stress.

545 6.7 Breeding Approaches for Improving Tolerance
546 to Combined Abiotic Stresses

547 Selection for one abiotic stress tolerance in the field is very challenging due to in-
548 teractions among the different stresses. Thus, the only strategy to identify the traits
549 to be applied in field for breeding tolerant genotypes is by performing experiments

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under controlled environment conditions. Regardless of the screening method, a key objective for plant breeders is to develop an effective set of stress combination markers that can be used to improve legume crop species. Controlled environmental conditions allow the dissection of each one of different stress effect and the identification of principal targets affecting plant tolerance. Breeding for stress tolerance requires efficient screening procedures, identification of key traits in diverse donor or tolerant lines and understanding their inheritance and molecular genetics. Statistical package applied to plant breeding will facilitate the identification of markers in a multi-trait multi-environment way (Malosetti et al. 2004).

AQ6

Several quantitative trait loci (QTL) studies relating to various abiotic stress tolerances have already been reported showing it is possible to improve and accelerate the breeding process in plant species without sequenced genomes (Chandra et al. 2004). In order to transfer these traits, classical breeding requires the establishment of rapid and cost-effective screening procedures and implementing these using breeding approaches such as association mapping or genomic selection procedures.

For the complete sequencing of the different important legumes, genome opens the possibility of fine mapping of the QTLs. In this perspective, gene identification for combined stress tolerance in legumes using genetic map information and genome data is an achievable goal (Heffner et al. 2009; Hirayama and Shinozaki 2010).

Phenotypic and physiological characterization along with RNA sequencing analysis of plants subjected to drought, heat, salt, flooding stress or their combination would confirm that the simultaneous imposition of different types of stress presents unique but varied aspects that includes alteration of respiration rate, decreased photosynthesis, stomatal closure, high leaf temperature and redox homeostasis. Thus, deep phenotyping methodologies, genome-based selection and massive RNA sequencing technologies emerge as a promising avenue for the development of multiple abiotic stress-tolerant crops.

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II. Respuestas a sequía en una leguminosa modelo

1. Uso de un transgénico para evaluar la funcionalidad de la prolina

El utilizar mutantes o silenciantes para genes que participan en la biosíntesis de prolina es una aproximación más concreta que la comparación de distintas especies para evaluar la participación de esta molécula en el estrés. Para eso en nuestro laboratorio se generó un transgénico de *L. japonicus* (Sainz et al., 2008) con el fin de silenciar un gen *p5cs* y de este modo impedir la acumulación de prolina. La secuencia que se insertó para generar el RNAi fue obtenida a partir de un *blast* de una secuencia p5cs de *Arabidopsis thaliana* sobre el genoma de *L. japonicus*.

Materiales y Métodos

Material vegetal

Las semillas de *L. japonicus* (Regel) Larsen cv. Gifu fueron cedidas por la Ing. Agr. M. Rebuffo, INIA La Estanzuela, Uruguay. Las 12 líneas transgénica silenciantes de *L. japonicus* fueron cedidas por la Dra. Martha Sainz (Sainz et al., 2008). Los transgénicos llevan una construcción para generar un RNAi del gen *p5cs*, bajo el promotor constitutivo 35S del virus del mosaico del coliflor. En la figura 6 se esquematiza la construcción insertada en el plásmido.

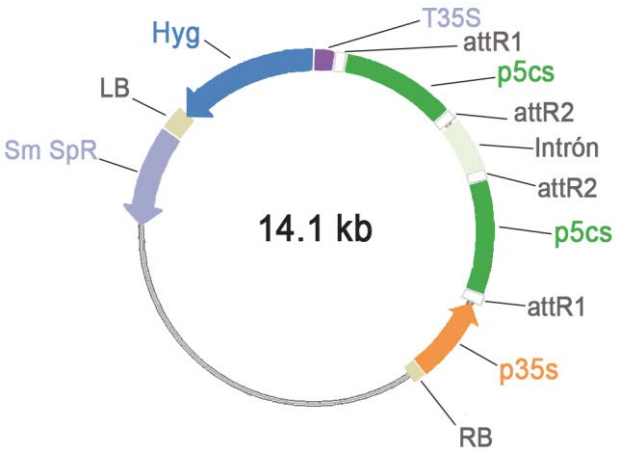

Figura 6. Construcción de silenciamiento. El vector binario pH7GWIWG2(I),0 lleva fragmentos del gen *p5cs* de *L. japonicus* bajo el promotor fuerte 35S, genes de resistencia a higromicina (Hyg), estreptomicina (Sm) y espectinomicina (SpR). LB y RB (borde izquierdo y derecho del transposón respectivamente) y el intrón de la chalcona sintasa.

Condiciones de crecimiento

Las semillas se esterilizaron superficialmente en alcohol 96° GL durante 1 min, con un enjuague con agua estéril, seguido por 3 min en hipoclorito de sodio al 20% (v/v), y 5 lavados sucesivos de 5 min en agua estéril. Las semillas germinaron a 25°C por 5 días en placas de Petri con agar-agua al 0,7 %, estéril. Las plantas crecieron en macetas que contenían arena:vermiculita (1:1) y solución nutritiva Rigaud y Puppo (Rigaud and Puppo, 1977) estéril cuya composición aparece a continuación. Las plantas crecieron durante 35 días en condiciones controladas con ciclos de 18/8 h luz/oscuridad y una densidad de flujo de fotones de 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

La solución Rigaud y Puppo (1977) se preparó de la siguiente forma:

Macroelementos	(g/L)	Microelementos	(mg/L)
K_2SO_4	0,174	EDTA-Fe	25
KH_2PO_4	0,068	$Na_2MoO_4.2H_2O$	0,11
K_2HPO_4	0,044	H_3BO_3	2,85
$MgSO_4.7H_2O$	0,123	$CuSO_4.5H_2O$	0,2
$CaSO_4$	0,120	$ZnSO_4.7H_2O$	0,55
KNO_3	0,505	$MnSO_4.H_2O$	3,07

Se ajustó a pH 7,5.

Imposición de déficit hídrico

El déficit hídrico se impuso por inmersión de los foliolos en una solución de polietilenglicol 8000 (PEG) al 30% en agua destilada (p/v). Esta solución genera un potencial hídrico de -1,5 Mpa. Como control se incubaron foliolos en placas de Petri con agua destilada. Los foliolos se mantuvieron durante 18 y 24 h en ambas condiciones, y se lavaron con agua destilada para remover el PEG inmediatamente antes de realizar las extracciones de ARN y prolina.

Extracción y cuantificación de Prolina

La extracción se realizó según (Chares and Phan, 1990) con modificaciones. Los foliolos se congelaron en nitrógeno líquido y se molieron en mortero. Se tomaron entre 50-100 mg de muestra y se maceró en microtubos con 800 µL de metanol-cloroformo-agua (MCA, 12:5:1). El homogeneizado se centrifugó a 5.000 x *g*

2 min a temperatura ambiente. Al sobrenadante se le adicionó 200 µL de cloroformo, 300 µL de agua destilada y se centrifugó a 5.000 x *g* 1 min. La fase superior (acuosa) se usó para la determinación de prolina, que se determinó según (Troll and Lindsley, 1955) con modificaciones. La totalidad del volumen de la fase acuosa se pasó a un tubo con tapa y se agregó 1 mL de ácido acético y 1 mL de ninhidrina ácida, consistente en H_3PO_4 6 M, ácido acético 12,6 M y ninhidrina 0,14 M. Se calentó en baño de agua a 90 °C durante 1 h y se dejó enfriar a temperatura ambiente. Seguidamente se adicionó 1 mL de tolueno, se agitó vigorosamente durante 5 min y se dejó decantar aproximadamente 12 h, hasta la formación de dos fases. Para cuantificar la prolina se tomaron muestras de la fase superior y se leyó la absorbancia a 515 nm. Como blanco se utilizó tolueno. La curva patrón se construyó con soluciones de prolina entre 0 y 120 nmoles · mL^{-1}. Las determinaciones se realizaron por triplicado de cada réplica biológica.

Expresión de genes por qRT-PCR

Los foliolos congelados en nitrógeno líquido se molieron en mortero y el ARN se extrajo según las instrucciones del fabricante del kit "*Isolate RNA kits"* (Bioline) para ARN de plantas.

Para eliminar el ADN las muestras se trataron con DNAsa (*New England Biolabs*). Para ello se resuspendió 10 µg de RNA en 1X "*DNase I Reaction Buffer"* hasta un volumen final de 100 µL. Se agregó 2 unidades de DNAsa, se mezcló suavemente y se incubó a 37° C 10 min. Se agregó 1 µl de EDTA 0,5 M (concentración final, 5 mM) y se incubó a 75° C durante 10 min para inactivar la DNAsa. Para la obtención de cDNA a partir del RNA se siguieron las instrucciones del fabricante del kit "*SuperScript™ III First-Strand Synthesis System for RT-PCR*" (Invitrogen).

Para la PCR en tiempo real cuantitativa (qRT-PCR) se siguieron las instrucciones del fabricante del kit "*QuantiFast SyBR Green RT-PCR*" (QIAGEN).

Los primers utilizados fueron:

LjUBC4 (*housekeeping*)
chr5.CM0956.27F TTCACCTTGTGCTCCGTCTTC
chr5.CM0956.27R AACAACAGCACACACAGACAATCC

LjP5CS1
chr1.CM0147.99F CCCGTGGCCCTGTAGGAGT
chr1.CM0147.99F TCCCGTCCCTCTCAATATCCA

LjP5CS2
chr2.CM0011.16F GACAGTGCGGCTGTTTTTCAC
chr2.CM0011.16R GTCCGAATAGTGCCCCATCA

Análisis estadístico

Se realizó el análisis de varianza de los resultados utilizando el test de Tukey's a un nivel de $p \leq 0,05$.

Resultados y Discusión

Identificación de potenciales secuencias *p5cs* en el genoma de *L. japonicus*

Para estudiar la expresión de los genes *p5cs* en *L. japonicus* primero se debió analizar las posibles proteínas P5CS en la base de datos. Para ello se identificaron en el NCBI (*National Center for Biotechnology Information)* las secuencias proteicas para P5CS A y P5CS B de *A. thaliana*. Estas secuencias se alinearon con las secuencias proteicas de *L. japonicus* a través de un *Blastp* y se obtuvieron 10 secuencias para cada *Blastp* (ver apéndice). Estas 10 secuencias eran las mismas en ambos blastp y sólo diferían en el score de similitud. A partir de las secuencias proteicas se buscó la secuencia de nucleótidos en el ADN que codificaba para estas proteínas, así como su localización cromosómica. Al analizar las secuencias se observó que 8 de ellas eran EST que pertenecían a las otras 2 secuencias encontradas con mayor score de similitud (ver apéndice). A la secuencia de *L. japonicus* que alineó mejor con la *p5cs A* inducible de *A. thaliana* se la llamamos *Ljp5cs* 1 y a la que alineó mejor con la *p5cs B* constitutiva de *A. thaliana* la llamamos *Ljp5cs 2*. Estos resultados indican que cada gen *p5cs* de *A. thaliana* tiene su homólogo en *L. japonicus*. Para cuantificar el nivel de expresión de estos genes por qRT-PCR se diseñaron primers específicos. En el apéndice se encuentran las secuencias de las proteínas y la secuencia de nucleótidos de *Ljp5cs 1* y *Ljp5cs 2* de *L. japonicus*.

Expresión de genes *p5cs* en plantas transgénicas

De las distintas líneas transgénicas construidas para silenciar el gen *p5cs* por RNAi, se seleccionaron 3 líneas para este análisis. Dos de ellas fueron elegidas por ser las que acumulaban menos prolina (líneas 15 y 16) y la otra por ser de las pocas líneas cuyas plantas no presentaban problema para producir semillas (línea 33), lo que permitiría seguir trabajando con ella.

Los foliolos de las líneas seleccionadas y del salvaje se sometieron a déficit hídrico impuesto por PEG 30 % y se determinó la concentración de prolina acumulada a 18 y 24 h. La figura 7 muestra el aumento de la concentración de prolina a las 18 y 24 h después de imponer el déficit hídrico.

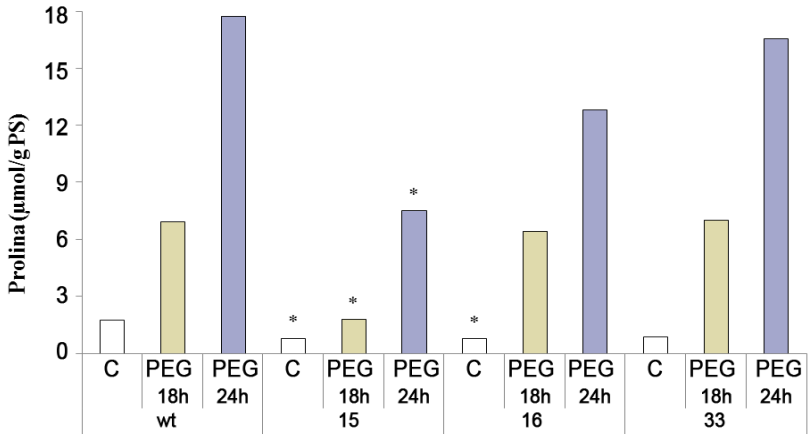

Figura 7. Concentración de prolina en foliolos. Condición control (C) y déficit hídrico (PEG) a 18 y 24 h. wt, salvaje; 15, 16 y 33 líneas transgénicas. Los asteriscos indican diferencias significativas con el wt correspondiente al mismo tratamiento, para 3 medidas de una misma réplica biológica.

A partir de los datos obtenidos, se decidió estudiar los niveles de expresión de las 2 *p5cs* encontradas en *L. japonicus* en control y PEG a las 24 h. Los valores de expresión de los genes para la P5CS 1 y la P5CS 2 se muestran en la figura 8. Como se puede observar el gen *Ljp5cs1* es inducible por déficit hídrico produciéndose un incremento en la expresión de 4 veces. En las líneas transgénicas también se indujo ese gen, sin embargo las cantidades fueron menores en ambas condiciones. Por lo tanto y como se esperaba, la P5CS 1 de *L. japonicus,* homóloga a la P5CS A de *Arabidopsis*, también es inducible.

La P5CS 2 no es inducible por déficit hídrico, ya que tanto en el salvaje como en las líneas transgénicas su expresión no se incrementó en PEG 30 % (Fig. 8). Esto se podía predecir porque el gen con mayor similitud en *A. thaliana* también es constitutivo. A su vez se observó que los valores de expresión en las líneas transgénicas son muy inferiores a los del salvaje, lo que evidencia que el RNAi silenció efectivamente el gen.

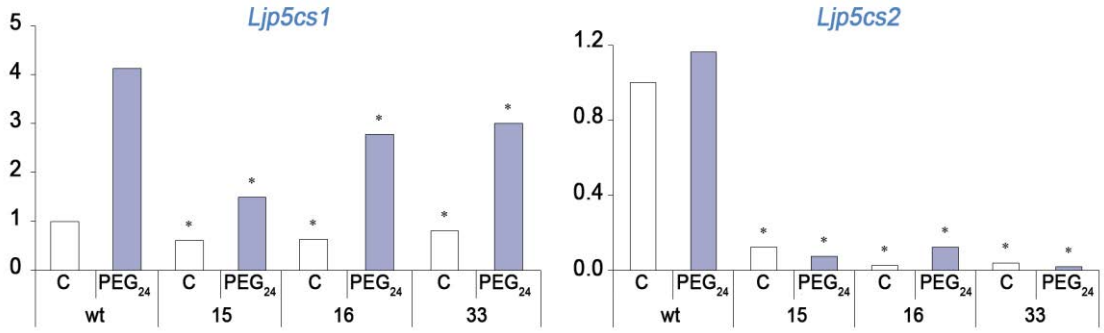

Figura 8. Expresión de los genes *Ljp5cs1* y *Ljp5cs2*. La expresión se determinó en condición control (C) y déficit hídrico a las 24 h (PEG_{24}). Los asteriscos indican diferencias significativas con el wt correspondiente al mismo tratamiento, para 3 medidas de una misma réplica biológica.

La tabla 1 muestra el porcentaje de silenciamiento obtenido en cada línea. En *Ljp5cs2* el silenciamiento promedio fue del 94 % tanto en condición control como en PEG 24 h. Esos valores de silenciamiento son mayores a los obtenidos para *Ljp5cs1*, que llegó a un 32 % en situación control y un 41% en PEG 24 h. Esta diferencia se debió a que la secuencia del RNAi usado era la del gen *Ljp5cs2*, por lo que silencia eficientemente a este gen, pero en menor grado al gen *Ljp5cs1* ya que tiene una similitud del 80%.

Tabla 1. Silenciamiento de los genes p5cs1 y p5cs2 en las transgénicas.

		% Silenciamiento			
		Ev.15	**Ev.16**	**Ev.33**	**Media**
Ljp5cs1	**CRL**	39.7	36.3	31.7	31.7
	PEG 24	63.9	19.1	41.3	41.3
Ljp5cs2	**CRL**	87.7	97.5	95.9	93.7
	PEG 24	93.6	89.2	98.2	93.7

Si se compara la expresión de los dos genes *Ljp5cs* se observa que la expresión de *Ljp5cs1* es mayor al de *Ljp5cs2*, figura 9. Esto hace que el efecto del silenciamiento

sea poco relevante, ya que no solo se silencia el gen constitutivo, sino que es el gen que menos se expresa de ambos. De esta forma, se pudo concluir que la enzima P5CS1 en *L. japonicus* es la principal responsable de la acumulación de prolina y por esto los transgénicos también acumularon prolina.

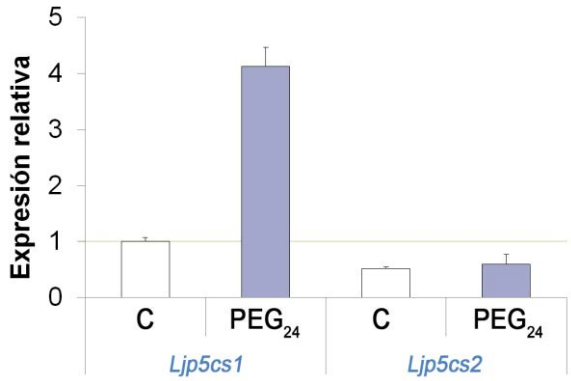

Figura 9. Expresión de *Ljp5cs1* y *Ljp5cs2*. Las expresiones son relativas al control de *Ljp5cs1*. C, Control; PEG_{24}, PEG 30% durante 24 h.

Estos resultados forman parte de un capítulo de libro que figura a continuación (Díaz et al., 2014).

Conclusiones

- *L. japonicus* cuenta con 2 genes *p5cs*, uno inducible al que denominamos *Ljp5cs1* y otro constitutivo al que denominamos *Ljp5cs2*.
- La imposición de estrés osmótico por PEG al 30 % durante 24 horas es suficiente para producir la inducción del gen *Ljp5cs1* y este es el responsable de la acumulación de prolina observada en ese periodo.
- Una similitud del 82% entre la construcción de RNAi y el gen a silenciar no es suficiente para obtener un silenciamiento adecuado.
- Estas líneas transgénicas de *L. japonicus* no son adecuadas como herramienta para explicar el rol de la prolina en respuesta a estrés.

4 Amino Acids and Drought Stress in Lotus: Use of Transcriptomics and Plastidic Glutamine Synthetase Mutants for New Insights in Proline Metabolism

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4.1 The Relationship between Nitrogen Metabolism and Drought Stress

Legumes are second only to the Graminae in importance to humans. In fact, legumes are widely used as a source of food, feed for livestock and raw materials for industry. Unfortunately, cultivated legumes are poorly suited for genomic research since several of them are tetraploids, have large genomes and are recalcitrant to transformation. For this reason, *Lotus japonicus* and *Medicago truncatula*, that have smaller and simpler genomes, have been adopted as models for legume research (Graham and Vance, 2003; Udvardi *et al.*, 2005; Stacey *et al.*, 2006). One of the ideas behind the use of model legumes is to exploit the synteny between model and cultivated species. This approach requires that the target genome region should contain the same genes in approximately the same position in model and crop species. Recent advances in comparative genomics confirm that the discoveries produced in *M. truncatula* and *L. japonicus* can frequently be extended to other legumes, including most members of the large and agriculturally important Papilionoid subfamily (Young *et al.*, 2005). Moreover, model legumes have the particular attraction that they can carry out a symbiotic relationship with nitrogen-fixing bacteria (Udvardi *et al.*, 2005; Stacey *et al.*, 2006); something that is not possible for other well exploited model species such as *Arabidopsis thaliana*. Since the beginning of the work with *L. japonicus* (Handberg and Stougaard, 1992), a great amount of physiological, biochemical and genetic studies have been carried out using this model legume (Márquez, 2005; Udvardi *et al.*,

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Table 4.1. *In vitro* nitrate reductase (NR) activity and nitrate concentration in *L. corniculatus* roots.

	Control	Drought stress
NR+EDTA[a]	100 ± 10	40 ± 8
NR+Mg^{2+} [a]	90 ± 11	35 ± 15
Nitrate concentration[b]	160 ± 10	170 ± 9

NR activity was determined in the presence of either EDTA or Mg^{2+}. Data are the mean ± standard deviation of three biological replicates. [a] Expressed in µmol NO_2^- mg^{-1} prot. h^{-1}. [b] Expressed in µmol NO_3^- g^{-1} DW. Adapted from Sainz (2006).

2005). The use of *L. japonicus* is of particular interest, and also *L. filicaulis and L. burtii* as models for the study of a number of cultivated *Lotus* species of great economic importance such as *L. corniculatus*, *L. uliginosus*, *L. tenuis* and *L. subbiflorus*, which have been domesticated and improved by plant breeding (Blumenthal and McGraw, 1999; Díaz *et al.*, 2005a; LOTASSA, 2010). This should promote the isolation and comparative characterization of genes from the less characterized cultivated *Lotus* species (LOTASSA, 2010; Castillo *et al.*, 2012).

Two of the factors that mostly limit the productivity of *Lotus* are drought stress and nitrogen availability (Borsani *et al.*, 1999; Márquez *et al.*, 2005). These two limiting factors are strictly interconnected. In fact, the metabolic pathways for nitrate and ammonium assimilation are affected by drought stress. Nitrate is reduced to ammonia by a two-step process catalysed by the enzymes nitrate reductase (NR) and nitrite reductase. Results from our groups have shown that drought stress results in a decrease of NR activity in *L. corniculatus* leaves and roots. However, NR activation state and nitrate content in both organs were not affected under the same conditions. As shown in Table 4.1, the presence of either Mg^{2+} or EDTA did not affect NR activity level under both control and drought conditions. This could indicate that the interaction between NR and 14-3-3 proteins was not modified by drought.

The main route for the assimilation of ammonia is the glutamine synthetase (GS)/glutamate synthase (GOGAT) pathway, while a minor assimilatory role may be played by glutamate dehydrogenase (GDH). The enzymes for ammonium assimilation were differently affected by drought stress in *Lotus*. Western blot analysis showed that drought stress resulted in increased levels of the cytosolic GS (GS1) isoform but did not affect the levels of the plastidic GS (GS2) polypeptide in *L. corniculatus* leaves (Fig. 4.1). On the other hand, the total GS activity was not modulated by drought in *L. japonicus*. This was determined by the quantification of the synthetase, transferase and biosynthetic activities of GS. However, the GS2 polypeptide content decreased while the GS1 content was increased in this *Lotus* species (unpublished results). The increase in GS1 content could be related with the re-assimilation of the ammonium derived from collateral proteolysis processes that are induced by drought (Skopelitis *et al.*, 2006). Most of the work carried out in leaves has not defined the specific contributions of GS1 and GS2 to total GS activity under osmotic stress conditions (Lutts *et al.*, 1999; Santos *et al.*, 2004) and, depending on the plant species analysed, total GS activity may decrease, increase or be unaffected by drought (Bernard and Habash, 2009).

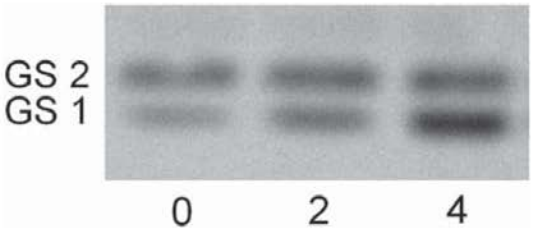

Fig. 4.1. Western blot analysis of GS in *L. corniculatus* leaves. The days spent by the plants under drought stress conditions are indicated.

A similar pattern of response to drought in different *Lotus* species was observed for Fd-GOGAT. The Fd-GOGAT activity was increased in response to drought in nodulated and nitrate-fed *L. corniculatus* plants (Borsani *et al.*, 1999; Díaz *et al.*, 2005c) while in *L. japonicus* Fd-GOGAT activity was increased in nitrate-fed plants but not in ammonium-fed plants under water deprivation (Díaz, 2011). This indicated that the modulation of Fd-GOGAT activity under drought is related with the nitrogen source available to the plant.

A role for GDH in ammonium assimilation has been proposed by several groups (Masclaux-Daubresse *et al.*, 2002; Skopelitis *et al.*, 2006). The authors suggested that the GDH aminating activity may be important during senescence and under abiotic stress conditions, situations where the ammonium concentration may be increased. In these conditions GDH may assimilate the ammonium produced, acting as a detoxification system. GS, GOGAT and GDH contribute to the maintaining of the glutamate and glutamine pools, which are the precursor of several compounds that accumulate under abiotic stress. For example, proline, gamma amino butyric acid (GABA) and asparagine are increased under water deprivation (Chiang and Dandekar, 1995). The metabolic pathways implicated in the synthesis of these molecules are drought-responsive and are highly dependent on glutamate and glutamine metabolism. Several of these compounds are called compatible solutes: small molecules that play several protective roles such as to maintain cell turgor and to scavenge reactive oxygen species (ROS) (Bartels and Sunkar, 2005).

4.2 Proline and Drought Stress in Lotus

Four *Lotus* species have been domesticated and improved by plant breeding: *L. corniculatus*, *L. uliginosus*, *L. tenuis* and *L. subbiflorus* (Blumenthal and McGraw, 1999; Díaz *et al.*, 2005a). All these cultivated species, as well as the models *L. japonicus*, *L. burtii* and *L. filicaulis* accumulate high levels of proline derived from *de novo* synthesis in response to drought stress (Díaz *et al.*, 2005c). On the other hand, in *L. corniculatus* leaves the decrease in proline degradation also contributes to the accumulation of this amino acid under drought (Borsani *et al.*, 1999). Proline is one of the most common compounds produced in plant cells in response to different kinds of abiotic stresses (Szabados and Savouré, 2010). Multiple roles have been proposed for this amino acid that may explain this conserved response. Among the roles proposed for proline are the ones of compatible osmolyte, C-N storage compound for recovery after stress, scavenger of ROS, regulator of redox potential and modulator of gene expression between others (Szabados and Savouré, 2010; Vendruscolo *et al.*, 2007). Proline also appears to be a very good marker of drought stress in several *Lotus* species. In fact, a strong correlation between proline accumulation and hydric deficit was observed in *L. japonicus* and *L. corniculatus* (Díaz *et al.*, 2005d; Díaz *et al.*, 2010). In *Lotus*, proline accumulation is detected at early stages of drought imposition, when the variation in tissue water content is yet very low. Increases of 20% in hydric deficit (HD) are enough to trigger proline accumulation in *Lotus* plant tissues (Table 4.2). For this reason, we propose that proline may act as an early and sensitive drought stress indicator in *Lotus*. Moreover, proline accumulation is also a common response to salt stress in different *Lotus* species (Sánchez *et al.*, 2011a).

The combination of drought and heat stress has a synergistic effect on proline accumulation in *L. japonicus* plants (Sainz, 2009). Similar results were obtained with *L. corniculatus* and *L. uliginosus*. Moreover, interspecific hybrids obtained by Castillo *et al.* (2012) using these last two species as parents showed a synergistic effect of drought and heat stress on proline accumulation.

It has been observed that proline content is affected by the nitrogen source available to the plants. In fact, *L. corniculatus* plants grown with ammonium accumulated at least twice as much proline than nodulated or nitrate-fed plants (Borsani *et al.*, 1999; Díaz *et al.*, 2005c). A similar dependence of proline accumulation from the nitrogen source under drought conditions has been observed also in *L. japonicus*. Moreover, *L. corniculatus* roots and *L. japonicus* nodules also accumulated proline in response to drought and salt stress respectively (López *et al.*, 2008; Sainz, 2009).

The content of other molecules related to proline metabolism, like hydroxyproline and pyroglutamate was also increased in WT *L. japonicus*

Table 4.2. Hydric deficit (HD) and proline concentration (PC) in cultivated and model *Lotus* species under drought stress.

	Control		Drought stress	
Genotype	HD[a]	PC[b]	HD	PC
L. corniculatus	20 ± 5	5.5 ± 1.5	41 ± 5*	65.0 ± 7.3*
L. tenuis	19 ± 4	4.0 ± 1.3	43 ± 4*	57.9 ± 5.1*
L. japonicus	21 ± 3	10.0 ± 2.5	38 ± 6*	61.2 ± 3.5*

[a] calculated as [turgid weight – fresh weight] / [turgid weight – dry weight] x 100.
[b] expressed in µmol g^{-1} dry weight. The results are mean of three replicates.
* indicate significant differences between control and drought stress at 5% Tukey's test

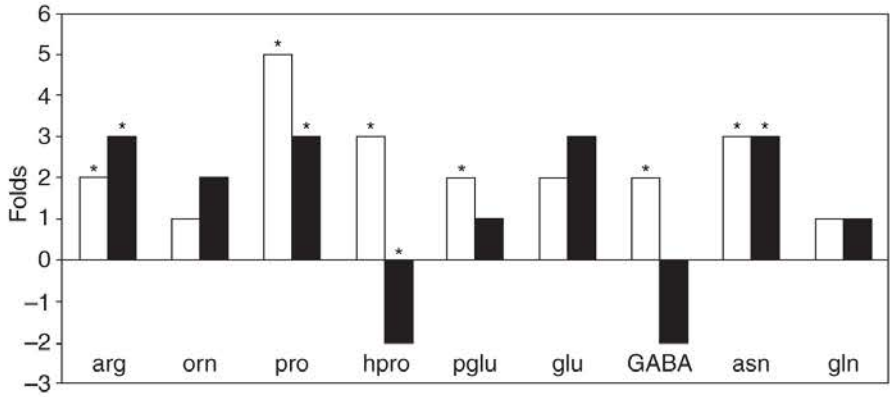

Fig. 4.2. Changes in nitrogen compounds under drought stress in WT (white bars) and *Ljgln2-2* mutant plants (black bars). Values indicate the fold-change of metabolite levels under drought stress with respect to the control. The compounds quantified by GC-MS were arginine (arg), ornithine (orn), proline (pro), glutamine (gln), gamma-aminobutyric acid (GABA), asparagine (asn), glutamate (glu), hydroxyproline (hpro) and pyroglutamate (pglu). The results are average of at least three repetitions. *Means significant difference with the control at 5% according to Student's t test. (Adapted from Díaz, 2011.)

plants under drought stress conditions (Fig. 4.2, white bars). These molecules could be produced by the reaction of proline with ROS (Matysik *et al.*, 2002). However, Sánchez *et al.* (2008) found that while proline concentration increases, hydroxyproline concentration decreased in salt-stressed *L. japonicus* plants. This indicates that different kinds of abiotic stress have different effects on proline metabolism.

Recently, a novel connection between plastidic glutamine synthetase and proline biosynthesis under drought stress was discovered by our group (Díaz *et al.*, 2010). For this reason, it was of particular interest to determine also the content of several molecules related to proline metabolism in response to drought in the *L. japonicus* plastidic GS mutant *Ljgln2-2* (Orea *et al.*, 2002; Betti *et al.*, 2006, 2012). The levels of proline in the mutant were lower than the WT in response to drought (Fig. 4.2, black bars), confirming that plastidic GS is important in supporting the stress-induced biosynthesis of proline. On the other hand, hydroxyproline content diminished in the mutant as a consequence of water deprivation. This was surprising since hydroxyproline is produced by the reaction of proline and hydroxyl radicals (Matysik *et al.*, 2002), and the *Ljgln2-2* mutant showed higher levels of oxidative stress under drought compared to the WT (Díaz *et al.*, 2010). Further metabolic studies should be carried out in order to clarify the role of this metabolite under drought conditions in the plastidic GS mutant.

The lower proline levels observed in the mutant under drought were accompanied by small or no changes in hydroxyproline and pyroglutamate concentrations respectively (Fig. 4.2). This indicates that GS2 deficiency, not only affects proline synthesis, but also the metabolites related to it.

The plastidic GS isoform produces glutamine, precursor for proline biosynthesis in the plastid, whereas cytosolic GS is responsible for the same contribution in the cytoplasm (Szabados and Savouré, 2010). The *Ljgln2-2* mutant has normal levels of GS1 polypeptide and activity (Orea *et al.*, 2002), so the glutamine for proline biosynthesis is probably provided by GS1 (Díaz *et al.*, 2010). Nevertheless, glutamate, glutamine and ornithine concentrations, precursors of proline synthesis, were not changed both in the *Ljgln2-2* and the WT genotype under drought stress (Fig. 4.2). For this reason, the minor proline accumulation observed in the mutant could not be assigned to precursor deficiency.

4.3 Proline and Drought Stress in Lotus: Transcriptomic Analysis

Proline accumulation under stress may result from a stimulation of proline biosynthesis, an inhibition of its oxidation or both (Szabados and Savouré, 2010). Proline is mainly synthesized from glutamate by the sequential action of pyrroline-5-carboxylate synthetase (P5CS) and pyrroline-5-carboxylate reductase (P5CR) (Székely *et al.*, 2008). This pathway is present in both the cytosol and the chloroplast (Szabados and Savouré, 2010). Alternatively, proline can be produced from ornithine by the action of ornithine-δ-aminotransferase (OAT), following the conversion of arginine into ornithine by the action of arginase (Delauney *et al.*, 1993). The degradation of proline occurs in the mitochondria and involves oxidation to pyrroline-5-carboxylate (P5C) by proline dehydrogenase (PDH), and subsequent conversion into glutamate by pyrroline-5- carboxylate dehydrogenase (P5CDH) (Székely *et al.*, 2008 and references therein).

The expression of the genes for both proline biosynthesis and degradation was studied in the mark of a transcriptomic experiment using both WT plants and the previously mentioned *Ljgln2-2* mutant from *L. japonicus*. For this, we used the newly developed Affymetrix® Lotus1a520343 Genechip that includes a total of 52,000 probesets that encompassed the major part of the *L. japonicus* genes (a probeset is an oligonucleotide designed to measure the expression of a known or predicted sequence of mRNA). RNA was extracted from well-watered plants and drought-stressed plants (with a RWC of 65%) and the genes modulated by drought were identified using a statistical cut-off of < 5% and applying a false discovery rate (FDR). Transcriptional analysis of the response of the *L. japonicus* genes for proline metabolism showed that the main pathway that involves glutamate as precursor was induced under drought conditions in both genotypes (Table 4.3). In particular, the biosynthetic P5CS gene was highly induced, especially in the *Ljgln2-2* mutant. Similar results were obtained by Sánchez *et al.* (2008) for *L. japonicus* WT plants under different salt stress conditions. In the alternative pathway, the expression of the arginase gene (*LjArg*) was induced only in the mutant (Table 4.3). No changes in the expression levels of the *LjOAT* gene were detected by the Affychips (Table 4.3). However, quantification of the corresponding transcript by qRT-PCR revealed a 3-fold induction of *LjOAT* exclusively in the mutant (Díaz *et al.*, 2010). For the other genes of proline metabolism, data from Affychip and qRT-PCR analysis were in good agreement (Díaz *et al.*, 2010).

The results presented here suggest that the OAT alternative pathway is not contributing in a relevant way to proline accumulation in WT *L. japonicus* plants, but may support the main pathway in the plastidic GS mutant. This mutant in fact accumulates a lower amount of proline in response to drought (Fig. 4.2), probably as a consequence of its lower proline biosynthetic capacity due to the absence of plastidic GS. Moreover, the higher level of induction of the P5CS genes in the mutant under drought may represent another compensatory mechanism (Díaz *et al.*, 2010). The increase in *P5CS* gene expression may also indicate that this is a regulation point of the pathway, with minor incidence of the availability of the precursor glutamate and ornithine.

Among the genes for proline degradation, the *P5CDH* gene generally shows a basal expression that is induced by exogenous application of proline (Deuschle *et al.*, 2001). In *L. japonicus*, *P5CDH* gene expression was up-regulated in the

The data obtained with the *Ljgln2-2* mutant under drought stress (Díaz *et al.*, 2010), as well as studies in *L. corniculatus* using inhibitors of photosynthetic electron chain (Díaz *et al.*, 2005c) showed that about 50% of the total proline accumulated under drought was synthesized in the chloroplasts, while the other 50% is probably synthesized in the cytosol. In agreement with our data, Brugière *et al.* (1999) found that in tobacco plants submitted to salt stress about 50% of the proline accumulated depended on the cytosolic GS isoform. Moreover, studies carried out in *Arabidopsis* showed that proline biosynthesis under control conditions is mainly cytosolic, whereas this synthesis is chloroplastic under drought stress conditions (Székely *et al.*, 2008).

A detailed sequence analysis revealed that the eight probesets that compose the groups P5CS2 and P5CS3 defined by Díaz *et al.* (2010) corresponded to the same gene located on chromosome 1 of *L. japonicus*. From now on we will call this gene *LjP5CS1* since it is homologous to the stress-responsive *P5CS1* gene of *A. thaliana*. On the other hand, the probesets corresponding to the P5CS1 group defined by Díaz *et al.* (2010) corresponded to a gene located on chromosome 2, which we now name *LjP5CS2* since it is homologous to the constitutive *P5CS2* gene of *A. thaliana* (Strizhov *et al.*, 1997; Székely *et al.*, 2008). Expression analysis of these two genes in *L. japonicus,* shows that *P5CS1* expression is induced by osmotic stress while *P5CS2* is not (Table 4.4), in a similar way to the corresponding *A. thaliana* homologous genes. Also, the basal expression of the inducible gene is higher than the constitutive gene. The probesets selected for the expression analysis were chr1.CM147.99 and chr2.CM0011.16 for LjP5CS1 and LjP5CS2 respectively because these probesets lead to high PCR efficiency. This is probably due to the fact that these sequences have a 100% of identity with the chromosomal sequence, while the other probesets were designed based on EST sequences.

4.4 Plastid GS Deficiency and Drought Stress: Transcriptomic Analysis

The modulation of the expression of the genes for proline biosynthesis hinted to more widespread transcriptional changes. In fact, a total of 2608 and 7915 probesets were modulated by water deprivation in the WT and *Ljgln2-2* mutant respectively (Díaz *et al.*, 2010). These modulated genes were classified into functional categories using the program Genebins (Goffard and Weiller, 2007). It was found that about 40% of the elicited genes had a known function in both genotypes, while about 60% of them were unclassified (Figure 4.3). Among the genes with known function, several were involved in important processes for the response to drought like membrane transport (3% and 2% in WT and mutant respectively) and amino acid metabolism (12% and 10% in WT and mutant respectively), with the latter category including genes involved in compatible osmolyte biosynthesis, among others. The group of genes related to signal transduction (8% in both genotypes) is also worthy of consideration since it may include the genes related with stress perception and signalling.

Several photosynthetic genes were repressed in the plastidic GS mutant under drought conditions. This regulation may have important consequences on the chloroplast metabolism. GS2

Table 4.4. Expression of *P5CS* genes under osmotic stress in *L. japonicus*. The expression levels were standardized to the ones of the *P5CS1* gene under control conditions, taken as 1. Osmotic stress was established by incubating the leaves in 30% PEG 8000 (–1.5 MPa) for 24 h.

Gene	Probeset	Control	Drought stress
LjP5CS1	chr1.CM0147.99	1.00 ± 0.07	4.13 ± 0.34
LjP5CS2	chr2.CM0011.16	0.51 ± 0.04	0.60 ± 0.17

PCR conditions and primer sequences for the *LjP5CS1*, *LjP5CS2* and the housekeeping *LjUBC10* that was used as internal standard were described by Díaz *et al.* (2010). Only PCR reactions with efficiencies higher than 90% were considered. Data are the mean ± standard deviation of three biological replicates.

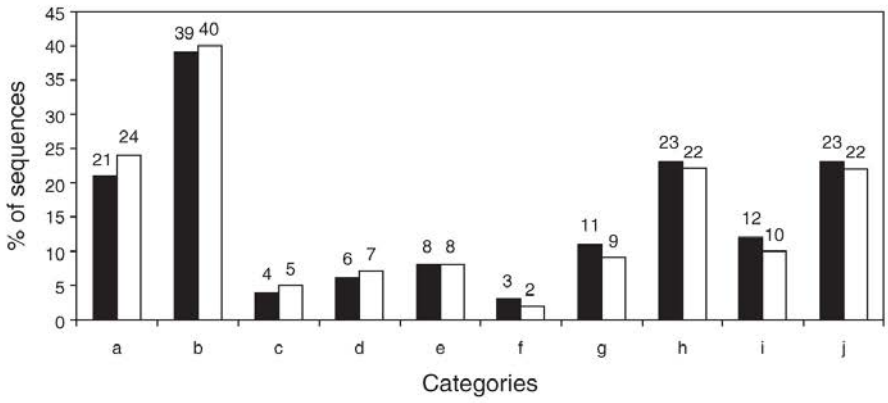

Fig. 4.3. Classification of the *L. japonicus* genes modulated by drought stress into non-redundant functional categories in the WT (black bars) and *Ljgln2-2* mutant plants (white bars). The functional classification was obtained using the Genebins program (Goffard and Weiller, 2007). a: Unclassified without homology; b: Unclassified with homology; c: Other cell functions; d: Cell motility, growth and death; e: Signal transduction and communication; f: Membrane transport; g: Replication, transcription and translation; h: Other metabolisms; i: Amino acids metabolism; and j: Nucleotides, carbohydrates and lipids metabolism.

Table 4.5. Expression of genes of the photorespiratory C2 cycle under drought stress in WT and *Ljgln2-2* mutant plants.

Gene	Probeset	Wild type	*Ljgln2-2*
C2 cycle			
LjGDCH2	Ljwgs_087246.1	**1.8**	**5.3**
LjGO1	Ljwgs_013523.1	**2.2**	**8.6**
LjGO2	Ljwgs_143630.1	**–1.9**	**–3.9**
LjHPR	Ljwgs_011418.2	**–1.7**	**–4.4**

The number represents the difference in relative expression levels of the reported genes between drought conditions and normal watering. Data are obtained from the mean of three independent hybridization of Affychips for each condition/genotype. Number in bold is the mean significant difference (FDR < 0.05). The genes reported encode for the H subunit of glycine decarboxylase (*LjGDH2*), two glycolate oxidase isoforms (*LjGO1* and *LjGO2*) and for hydroxypyruvate reductase (*LjHPR*).

deficiency was associated to a major repression of these genes, which is probably a symptom of higher levels of stress in this genotype (Díaz *et al.*, 2010). The minor proline concentration found in the mutant may be a reflex of lower plastidic proline biosynthesis in the absence of GS2. However, the repression of photosynthetic genes in the mutant would also result in a decrease of NADPH availability. Since high levels of NADPH are needed for massive accumulation of proline, the repression of photosynthetic metabolism in the *Ljgln2-2* mutant may also explain the lower accumulation of proline showed by this genotype. As was mentioned before, the application of propanil (3´4´- dichloropropioanilid) an inhibitor of photosynthetic electron flux to *L. corniculatus* leaves under osmotic stress conditions inhibited NADPH production. This resulted in a drop of proline accumulation of about 50%. This difference was assigned to the minor availability of reductant power derived from photosynthesis (Díaz *et al.*, 2005c).

Several genes of the photorespiratory C2 cycle were also induced in both genotypes, including glycine decarboxylase and the *LjGO1* isoform of glycolate oxidase, while others like hydroxypyruvate reductase and the *LjGO2* isoform of glycolate oxidase were repressed (Table 4.5).

This suggests a coordinate regulation of the photorespiratory genes under drought stress conditions in *L. japonicus*.

4.5 Nitrogen Nutrition and Proline Metabolism in Lotus

As mentioned before, several responses of *Lotus* species to water deficit depend on the nitrogen source available. In order to get further insight into this dependence, *L. japonicus* plants were cultivated with ammonium or nitrate as the unique nitrogen source. The plants were then subjected to drought stress by withholding watering and the transcript levels of the genes and enzymatic activities for proline metabolism and ammonium assimilation were quantified. Proline accumulation under drought conditions was observed both for the nitrate- and the ammonium-fed plants. However, proline levels under drought conditions were higher in the case of the ammonium-fed plants with respect to those of the nitrate-fed plants. In agreement with data obtained with other *Lotus* species, no changes in total protein and total amino acid were observed under both nitrogen nutritions (Díaz *et al.*, 2005b). The difference in proline levels observed between nitrate and ammonium-fed plants could not be assigned to a different water status since the RWC was the same for drought-stressed plants grown under nitrate or ammonium.

It was also observed that glutamate concentration decreased in response to drought stress in nitrate-fed plants but not in ammonium-fed plants. Under saline stress, Rhodes *et al.* (1999) and Sánchez *et al.* (2008) also observed a decrease of glutamate concentration and increase of proline concentration.

In order to explain the differential proline accumulation found in ammonium- and nitrate-fed plants, the activity of P5CS and OAT, the enzymes involved in the main and alternative proline biosynthetic pathway, was determined. A significant increase of P5CS activity in response to hydric deficit under both nitrogen regimens was observed (Fig. 4.4). Moreover, ammonium-fed

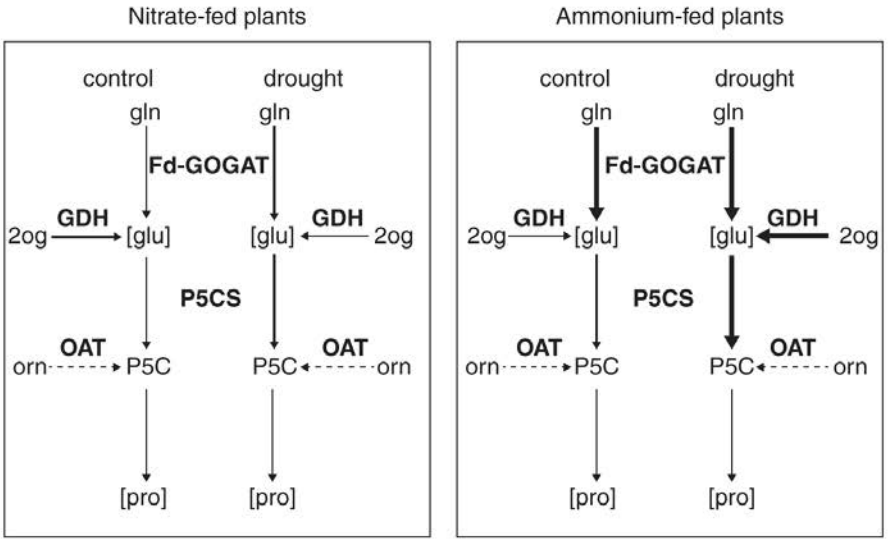

Fig. 4.4. Schematic representation of the change in the activity of the enzymes for proline biosynthesis in leaves of nitrate- and ammonium-fed *L. japonicus* plants subjected to drought stress. The activities of GOGAT, aminating glutamate dehydrogenase (GDH), ornithine amino transferase (OAT) and pyrroline 5 carboxylate synthetase (P5CS) were measured under control and drought conditions. Solid arrows represent enzyme activities that are induced by drought. The width of the solid arrows is proportional to the increase in the activity. Dashed arrows represent enzymes whose activity is not changed by drought. Also glutamate and proline concentration were determined. The font size indicates the different concentration of the amino acid.

plants showed higher P5CS activity with respect to the nitrate-fed ones (Díaz, 2011) under both control and drought conditions. On the other hand, OAT enzyme activity was not changed in response to drought under both nitrogen regimens (Fig. 4.4).

Under drought stress conditions, *Arabidopsis* plants grown under both nitrate and ammonium showed a clear regulation of the *P5CS1* gene (Székely *et al.*, 2008). In a similar way, the expression analysis in *L. japonicus* showed that under drought stress the *LjP5CS1* gene is induced by both nitrogenous sources while no changes were observed in *LjP5CS2* gene. The increase of the P5CS1 transcript under drought stress may lead to increased P5CS enzyme activity in order to produce proline from the glutamate derived by the GOGAT (Rhodes *et al.*, 1999) and GDH activities (Skopelitis *et al.*, 2006).

In *L. japonicus* nitrate-fed plants, Fd-GOGAT activity increased in response to drought stress, but the expression of the correspondent gene was decreased (Díaz, 2011). An increase in Fd-GOGAT activity was observed in different species in response to different types of stress (Berteli *et al.*, 1995; Borsani *et al.*, 1999; Díaz *et al.*, 2005c), and it might serve to ensure the supply of glutamate. Contrarily to what is observed for *L. japonicus*, in *Phaseolus vulgaris* (Becker *et al.*, 1986) and *Zea mays* (Sengar *et al.*, 1995) drought-stressed plants both GOGAT activity and gene expression were diminished. These results suggest that different mechanisms of regulation of GOGAT are operative in different plant species under abiotic stress (Srivastava *et al.*, 2002; Márquez *et al.*, 2005).

Another source of glutamate that should be taken into consideration is that derived from the aminating activity of GDH. The GDH aminating activity increased in response to drought stress in *L. japonicus* exclusively in ammonium-fed plants (Fig. 4.4). An increase in GDH aminating activity was also observed in tobacco plants and wheat seedling submitted to salt stress (Skopelitis *et al.*, 2006; Wang *et al.*, 2007). Additionally, Fd-GOGAT activity was higher in ammonium-fed than in nitrate-fed plants under both control and drought conditions (Fig. 4.4). Thus, it is possible that both GDH aminating and Fd-GOGAT activities may provide the glutamate necessary for proline accumulation in ammonium-fed plants. This is in agreement with the increased proline biosynthetic capacity under drought shown by the ammonium-fed plants with respect to the nitrate-fed ones.

Glutamine, one of the GOGAT substrates, is produced exclusively by GS. In ammonium- or nitrate-fed plants, GS activity was not changed as a consequence of drought (not shown). In agreement with this, the expression levels of the three genes encoding for cytosolic GS that were found in the *L. japonicus* genome were not significantly modulated by drought with the exception of *LjGS1-2*, which was slightly induced by drought in the mutant (Table 4.3). On the other hand, the *LjGln2* gene that encodes for the plastidic GS isoform was repressed under water deprivation. The results available in the bibliography about the response of GS activity to osmotic stress conditions do not allow the identification of a common pattern since changes in GS enzyme activity and gene expression are very species-specific (Bernard and Habash, 2009). However, from the data reported here it can be concluded that in ammonium-fed *L. japonicus* plants both the GS/GOGAT cycle and the aminating activity of GDH contribute to generate the glutamate for proline production. On the other hand the increase in Fd-GOGAT activity may support proline accumulation in nitrate-fed plants under drought conditions (Fig. 4.4).

Summarizing, the activity of the enzymes for nitrogen and proline metabolism, more than precursor availability seem responsible for the major proline accumulation observed in ammonium-fed plants. Additionally, we have presented evidence that supports the role of GDH to support proline biosynthesis under drought stress conditions, particularly under ammonium nutrition.

4.6 Role of Other Amino Acids in Drought Stress in *Lotus*

Proline is not the only nitrogen molecule that is accumulated by plants under abiotic stress. GABA is a ubiquitous, non-protein amino acid that accumulates rapidly in plants in response to environmental stress. GABA at high concentrations is able to stabilize and protect isolated thylakoids against freezing damage in the presence of salt, exceeding the cryoprotective properties of proline (Bouché and Fromm, 2004). In addition,

GABA possesses *in vitro* hydroxyl-radical scavenging activity that exceeds the ones of proline and glycine betaine at similar concentrations (Shelp *et al.*, 1999). However, this evidence comes from indirect experimental approaches.

A. thaliana mutants deficient in GABA-shunt are more sensitive to environmental stresses because they are unable to scavenge hydrogen peroxide (Bouché *et al.*, 2003). In *L. japonicus* plants, GABA concentration and glutamate decarboxylase (GDC) transcripts were increased under drought conditions (Fig. 4.2, Table 4.3). GABA was increased under drought stress also in *L. uliginosus* (Díaz *et al.*, 2005b).

The *LjGDC* gene was more up-regulated in *Ljgln2-2* mutant than in the WT under water deprivation (Table 4.3). Despite this, GABA concentration was lower in the mutant (Fig. 4.2). In the light of these results, it may be possible there exists a compensatory mechanism similar to that mentioned in the case of proline metabolism. As GABA could participate in the scavenging of ROS (Shelp *et al.*, 1999), the lower amount of GABA accumulated by the *Ljgln2-2* mutant may also explain the higher oxidative damage shown by this genotype under water deprivation (Díaz *et al.*, 2010).

The kinetics of GABA accumulation in plants reveals a stress-specific pattern that is consistent with a physiological role for GABA in stress mitigation. Stress results in cytosolic acidification and leads to an activation of GDC in a pH-dependent manner that results in increased GABA synthesis. Also a rapid increase of cellular levels of Ca^{2+} stimulates calmodulin-dependent GDC and therefore increases GABA synthesis. The different expression patterns of *GDC* genes and proteins suggest that GDC activity is transcriptionally and translationally regulated (Shelp *et al.*, 1999). Rapid accumulation of GABA in stressed tissue may provide a critical link in the chain of events leading from the perception of environmental stresses to timely physiological responses (Bouché and Fromm, 2004).

Asparagine is another nitrogen compound that accumulates in response to abiotic stress. Despite this, little attention has been paid to the accumulation of asparagine in comparison with proline and GABA. Some abiotic stresses enhance protein degradation and produce concomitant ammonium accumulation in the plant. Under such circumstances, the synthesis of free amino acids, and especially of the amide asparagine, is increased possibly to re-assimilate the nitrogen released in order to prevent ammonium toxicity (Herrera-Rodríguez *et al.*, 2007). Under severe drought stress asparagine increased markedly in soybean (Fukutoku and Yamada, 1984), *L. corniculatus* and *L. uliginosus* leaves (Díaz *et al.*, 2005b). Salt stress also leads to an increase of asparagine in *L. japonicus* MG20, *L. filicaulis*, *L. burtii* and *L. glaber* and was related with a higher tolerance to salinity. These results suggest that asparagine may play a pivotal role in salt tolerance by supporting core nitrogen metabolism (Sánchez *et al.*, 2011b).

The glutamine dependent asparagine synthetase (AS) enzyme is the major route for asparagine biosynthesis in plants. Therefore, a positive response of AS gene expression to stress conditions could be expected. The expression of sunflower AS genes is regulated and correlated with asparagine levels in tissues under osmotic stress (Herrera-Rodríguez *et al.*, 2004). Studies of gene expression under stress showed an increase in AS transcripts in barley and wheat under osmotic and salt stress respectively (Ueda *et al.*, 2004; Wang *et al.*, 2005).

Experiments carried out with the *Ljgln2-2* mutant revealed that the asparagine concentration increased in response to drought stress (Fig. 4.2). This indicates that the synthesis of asparagine is supported mainly by the glutamine produced by GS1. However, it has to be pointed out that since asparagine could be transported to other plant organs, increases in asparagine concentration may not necessarily result from increased gene expression or enzyme activity in the tissue analysed.

AS and asparaginase are responsible for maintaining the asparagine pool. In wheat and sunflower it was found that the AS genes are induced by osmotic stress (Wong *et al.*, 2004; Herrera-Rodríguez *et al.*, 2007) and this is considered a general response to the osmotic stress condition (Gaufichón *et al.*, 2010). On the other hand, one asparaginase gene was also induced by thermal or osmotic stress in soybean (Cho *et al.*, 2007). Transcriptomic analysis showed that in both WT and *Ljgln2-2* mutant the *L. japonicus* asparaginase *LjNSE2* gene was induced under the drought stress condition, whereas one of the two genes encoding for AS, *LjAS2*, was induced by drought only in the mutant (Table 4.6). This could

Table 4.6. Expression of genes of asparagine metabolism under drought stress in WT and *Ljgln2-2* mutant plants.

Gene	Probeset	Wild type	*Ljgln2-2*
Asparagine metabolism			
LjNSE1	chr5.CM0096.107	1.4	**2.8**
LjNSE2	Ljwgs_021574.1	**3.9**	**4.6**
LjNSE3	TM1930.50	n.d	n.d.
LjAS1	gi897770	1.4	1.9
LjAS2	gi897772	1.8	**2.9**

The number represents the difference in relative expression levels of the reported genes between drought conditions and normal watering. Data are obtained from the mean of three independent hybridization of Affychips for each condition/genotype. Number in bold is the mean significant difference (FDR < 0.05). The genes reported encode for three asparaginase isoforms (*LjNSE1*, *LjNSE2* and *LjNSE3*) and two asparagine synthetase isoforms (*LjAS1* and *LjAS2*). n.d.: not detected.

be a reflection of the major levels of stress perceived/received by the mutant with respect to WT. The differential response to drought stress found for AS and asparaginase genes, could be explained because these genes are expressed in distinct plant tissues (Waterhouse *et al.*, 1996; Credali, 2011; Credali *et al.*, 2011).

Asparagine is a major nitrogen transport and storage amino acid during germination, nitrate assimilation and nitrogen fixation in plants (Girousse *et al.*, 1996; Waterhouse *et al.*, 1996). Although it has been well documented that asparagine is implicated in the response of plants to abiotic stress, the precise function of this amino acid in stress remains unclear. Results from some studies suggested that asparagine may detoxify the ammonia produced under abiotic stress conditions (Herrera-Rodríguez *et al.*, 2007).

The concentration of compatible osmolytes and nitrogen compounds is not necessarily the direct result of gene expression or enzyme activity. Other regulatory actors that participate in the drought stress responses could be involved. The results exposed in this chapter evidence the necessity to realize genetic, biochemical and metabolic analyses simultaneously, in order to improve our knowledge about plants metabolic network under drought stress condition.

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2. Estrés nitro-oxidativo inducido por sequía en *L. japonicus*

En esta tesis se caracterizó el estrés nitro-oxidativo que se genera en las líneas transgénicas de *L. japonicus* y el salvaje con el fin de determinar si la prolina protege frente al daño oxidativo, como es considerado en la literatura. Los resultados no mostraron diferencias en el daño nitro-oxidativo ni en la respuesta antioxidantes entre las líneas transgénicas y el salvaje. De todas formas no se pudo descartar la participación de la prolina en la defensa antioxidante porque las líneas transgénicas igualmente la acumularon. Sin embargo, los resultados relacionados al estrés nitro-oxidativo inducidos por la sequía en el modelo *L. japonicus* son de interés y forman parte del próximo artículo que se presenta (Signorelli et al., 2013*b*). Este artículo incluyó la evaluación del estrés nitro-oxidativo en raíz, un órgano poco estudiado que podría tener un rol importante en la percepción de la sequía. Los resultados muestran que la sequía produce un estrés nitro-oxidativo donde el estrés nitrosativo es mayor en raíces que en hojas y el estrés oxidativo es mayor en hojas que raíces. Esto se observó posteriormente en plantas de tomate sometidas a estrés salino (Manai et al., 2014). En ambos órganos se evidenció la acumulación de prolina en respuesta a estrés, probablemente por la sobreexpresión del gen *Ljp5cs1* como se demostró en esta tesis. Las actividades de las enzimas GR, CAT y APX también se indujeron en ambos órganos a excepción de la APX en raíz cuya actividad disminuyó. Nuevas evidencias demostraron que la APX citosólica de arveja se inactiva al ser nitrada (Begara-Morales et al., 2014). Considerando esto y que determinamos nitración de proteínas en raíces de *L. japonicus*, es posible que el descenso de la actividad APX se deba a su nitración.

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Water stress induces a differential and spatially distributed nitro-oxidative stress response in roots and leaves of *Lotus japonicus*

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ABSTRACT

Water stress is one of the most severe problems for plant growth and productivity. Using the legume *Lotus japonicus* exposed to water stress, a comparative analysis of key components in metabolism of reactive nitrogen and oxygen species (RNS and ROS, respectively) were made. After water stress treatment plants accumulated proline 23 and 10-fold in roots and leaves respectively, compared with well-watered plants. Significant changes in metabolism of RNS and ROS were observed, with an increase in both protein tyrosine nitration and lipid peroxidation, which indicate that water stress induces a nitro-oxidative stress. In roots, •NO content was increased and *S*-nitrosoglutathione reductase activity was reduced by 23%, wherein a specific protein nitration pattern was observed. As part of this response, activity of NADPH-generating dehydrogenases was also affected in roots resulting in an increase of the NADPH/$NADP^+$ ratio. Our results suggest that in comparison with leaves, roots are significantly affected by water stress inducing an increase in proline and •NO content which could highlight multiple functions for these metabolites in water stress adaptation, recovery and signaling. Thus, it is proposed that water stress generates a spatial distribution of nitro-oxidative stress with the oxidative stress component being higher in leaves whereas the nitrosative stress component is higher in roots.

1. Introduction

Water stress is a common plant environmental stress that dramatically limits growth and development. Water stress can trigger a significant decrease in crop productivity and quality, and is especially evident in forage legumes since it accompanies loss in milk production and livestock. A number of different species of *Lotus* are widely used in dairy production and animal husbandry. While not as productive as clover or alfalfa, *Lotus* plants have higher animal nutrition values and very importantly do not provoke bloating in ruminants, as do other forage legumes [1]. *Lotus japonicus* (lotus) is a well-established model legume closely related to forage legumes such as *Lotus corniculatus*, *Lotus tenuis* and *Lotus uliginosus* [2,3]. These legumes are typically subjected to a variety of different environmental stresses such as high salinity, drastic temperature changes, high solar radiation, atmospheric pollution and water stress [4].

At the cellular level water stress induces overproduction of ROS, such as hydrogen peroxide (H_2O_2), superoxide radical ($O_2^{\bullet -}$) and hydroxyl radical (•OH), which are responsible for oxidative damage associated with stress [5,6]. Plants respond to stress using different enzymatic and non-enzymatic antioxidant systems. Previous studies have shown that these antioxidant defense systems are induced in water stress tolerant cultivars of wheat [7], tomato [8] or alfalfa [9], and the induction of these antioxidant systems was associated with water stress acclimation. Oxidative stress responses may involve increased activity of SOD, CAT and ascorbate–glutathione cycle activities such as GR or APX, which can confer greater tolerance against a specific environmental stress [10]. Increased levels of non-enzymatic soluble antioxidants including glutathione (GSH), ascorbic acid and tocopherols are also produced in response to water stress-induced oxidative stress [11]. Plant antioxidant defense systems normally provide adequate protection against ROS damage under optimal growth conditions. The generation of higher levels of ROS may overcome defense provided by these systems to result in oxidative stress [6,12,13]. Cellular damage caused by

Abbreviations: APX, ascorbate peroxidase; CAT, catalase; G6PDH, glucose-6-phosphate dehydrogenase; GR, glutathione reductase; GSNOR, *S*-nitrosoglutathione reductase; ICDH, isocitrate dehydrogenase; ME, malic enzyme; •NO, nitric oxide; 6PGDH, 6-phosphogluconate dehydrogenase; RNS, reactive nitrogen species; ROS, reactive oxygen species; SNOs, nitrosothiols; SNP, sodium nitroprusside; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.

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oxidative stress includes lipid peroxidation, which increases in various tissues during water stress and is also a commonly marker of oxidative stress [10].

RNS include •NO, nitrogen dioxide (•NO_2), *S*-nitrosothiols (SNOs) and peroxynitrite ($ONOO^-$), and accumulation of these species can result in nitrosative stress [14]. A great deal of free radical oxidizing damage is subjected to compartmentalization by lipid membranes, which offer significant resistance to the passage of most reactive species [15]. However, some of these molecules can diffuse freely across lipid membranes, like •NO [16] and •NO_2 [17], to react with low molecular weight antioxidants, unsaturated lipids and proteins in different compartments. Nitration of tyrosine residues can result in a change in protein function resulting in inactivation, i.e. CAT, MnSOD and GR [18,19], or gain of function, such as activation of the peroxidatic activity of cytochrome c [20]. Nitration of protein tyrosines has been established as a potential biomarker for nitrosative stress in plants [21,22]. Another modification mediated by RNS is *S*-nitrosylation of protein, and this process is of particular interest because it may enable reversible regulation of protein function as well as contribute to nitrosative stress [23–26]. Reduced glutathione is the most abundant intracellular non-protein thiol, and can easily be *S*-nitrosylated by •NO to form *S*-nitrosoglutathione (GSNO). The principal compounds thought to nitrosylate cellular proteins are thus nitric oxide and GSNO. GSNOR activity decomposes GSNO to GSSG and NH_3 and therefore can indirectly modulate •NO cell content [27]. Under different stress conditions such as biotic stress, heat stress, wounding, GSNOR decreases both at the level of gene expression and enzyme activity, thereby leading to increased cellular GSNO and SNOs [28–31].

There is very limited information available on plant metabolism of •NO under water stress conditions. Therefore, the main goal of our study is to analyze occurrence, distribution and metabolism of key components of both RNS and ROS under water stress using model legume *L. japonicus*. We also wished to address the issue of how these two classes of stress metabolites (ROS and RNS) might interact during water stress and acclimation for tolerance. Our results demonstrate that water stress results in a differential nitro-oxidative stress response in roots and leaves in lotus.

2. Materials and methods

2.1. Plant materials and growth conditions

L. japonicus (Regel) Larsen cv. Gifu seeds were obtained from Ing. Agr. M. Rebuffo, INIA La Estanzuela, Uruguay. Seeds were surface-sterilized first by immersion in absolute ethanol for 1 min, washed once with sterile water, followed by 3 min in sodium hypochlorite 20% (v/v), and finally five successive washes with sterile water. Seeds were germinated at 25 °C for 2 days. Plants were grown for 35 days under controlled conditions: 16/8 h light/dark cycle at 24/18 °C with photosynthetic photon flux density of 250 μmol m^{-2} s^{-1}. Plants were grown in Leonard jars containing a mix of sand:vermiculite (1:1) as substrate and supplied with Rigaud and Puppo nutrient solution supplemented with 1 mM KNO_3 [32]. Water stress was induced by removing the nutrient solution from the Leonard jars. The plants were kept in the jars, sealed with Parafilm® to prevent the excessive dehydration of the root system. The moisture in the jars declined slowly producing a slow water stress evident after 5 days of treatment. The weight of the pots were monitored during the treatment to determinate the humidity of the sand:vermiculite substrate. Relative humidity to weight (RHW) was determined as follows: RHW = (Weight of H_2O in the substrate × 100)/Dry weight of substrate [33]. At the moment of the sampling, the RHW was around 48% and 9%, in control and stressed plants, respectively.

2.2. Plant crude extracts

Sampled leaves were selected from the first to the seventh expanded leaf and in the root system, all lateral growing roots were used. Selected leaves and roots were frozen in liquid N_2 and ground in a mortar. The powder was suspended in a homogenizing medium composed of 50 mM Tris–HCl, pH 7.8, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol (DTT) and 0.2% (v/v) glycerol. Homogenates were centrifuged at 20,000 × *g* for 20 min (4 °C) and supernatant was used immediately for assays.

2.3. Enzyme assays

All the enzyme assays were performed from crude extracts. CAT (EC 1.11.1.6) activity was determined by measuring the disappearance of H_2O_2, as described by Aebi [34]. APX (EC 1.11.1.11) activity was determined by monitoring oxidation of ascorbate by H_2O_2 at 290 nm [35]. GR (EC 1.6.4.2) activity was assayed by monitoring oxidation of NADPH coupled to the reduction of GSH [36]. The reaction rate was corrected for the small non-enzymatic oxidation of NADPH by glutathione disulfide (GSSG). GSNOR (EC 1.2.1.1) activity was assayed spectrophotometrically at 25 °C by monitoring the oxidation of NADH at 340 nm [37]. G6PDH (EC 1.1.1.49) activity was determined spectrophotometrically by monitoring the reduction of NADP at 340 nm. Assays were performed at 25 °C in a reaction mixture (1 mL) containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.6, 2 mM $MgCl_2$ and 0.8 mM $NADP^+$. The reaction was initiated by the addition of 5 mM glucose-6-phosphate. For the determination of 6PGDH (EC 1.1.1.44) activity, the reaction mixture was like that described for G6PDH, but the substrate was 5 mM 6-phosphogluconate [38]. NADP-ICDH (EC 1.1.1.42) activity was also measured by following $NADP^+$ reduction, but including 10 mM 2R,3S-isocitrate as substrate [39]. NADP-ME (EC 1.1.1.40) activity was also determined by recording the reduction of $NADP^+$ using the same reaction mixture, but was initiated by addition of 1 mM L-malate [40]. One enzymatic unit was defined as the activity that consumed 1 μmol of cofactor or substrate per minute.

2.4. SOD isozymes

SOD isozymes present in crude extracts were separated by non-denaturing polyacrylamide gel electrophoresis (PAGE) on 10% acrylamide gels as described by [41], and activity was visualized using a photochemical nitroblue tetrazolium (NBT) reduction method [42]. To identify the type of SOD isozyme activity present in a gel, specific isozyme inhibitors were included in the activity stain. Gels were incubated separately at 25 °C for 30–45 min in 50 mM K-phosphate, pH 7.8, in the presence or absence of either 2 mM KCN or 5 mM H_2O_2. CuZn-SOD is inhibited by KCN and H_2O_2, Fe-SOD is inhibited by H_2O_2 but not by KCN, whereas Mn-SOD is not inhibited by either KCN or H_2O_2 [38].

2.5. Immune-detection of nitrotyrosine

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done according to the method of Laemmli [43] in 10% acrylamide slab gels. For Western blot analysis, proteins were transferred to PVDF membranes with a semi-dry Trans-Blot cell (Bio-Rad, Hercules, CA, USA). After transfer, membranes were used in cross-reactivity assays with a rabbit polyclonal antibody against 3-nitrotyrosine [28] diluted 1:10,000. For immuno-detection, an affinity-purified goat anti-(rabbit IgG)-horseradish peroxidase conjugate (Bio-Rad) and an enhanced chemiluminescence kit (ECL PLUS, Amersham, Piscataway, NJ, USA) were used. Commercially

prepared nitrated bovine serum albumin (BSA) was used as positive control (Sigma, St. Louis, MO, USA).

2.6. Spectrofluorometric detection of •NO

Freshly prepared crude extract suspensions of roots or leaves were mixed with 4,5-diaminofluorescein (DAF-2) to a final concentration of 10 μM. Reaction mixtures were incubated at 37 °C in the dark for 2 h, and fluorescence was measured in a QuantaMaster™ QM-4 fluorescent spectrophotometer (PTI® Photon Technology International, Lawrenceville, NJ, USA) at excitation and emission wavelengths of 485 and 515 nm, respectively [44].

2.7. Detection of •NO by confocal laser scanning microscopy (CLSM)

•NO was detected in lateral root tips with 10 μM 4-aminomethyl-2′,7′-difluorofluorescein diacetate (DAF-2 DA, Sigma, St. Louis, MO, USA) prepared in 10 mM Tris–HCl (pH 7.4) as described elsewhere [22]. Roots were examined with a confocal laser scanning microscope (Leica TCS SL, Leica Microsystems, Heidelberg GmbH, Wetzlar, Germany).

2.8. Relative water content

To determine the relative water content (RWC), fresh roots and leaves were weighed (FW), then these were hydrated with distilled water for 24 h and turgent weight was determined (TW), and finally these roots and leaves were dried at 80 °C in an oven and dry weight was determined (DW). The RWC was calculated as follows: $100 \times (FW - DW)/(TW - DW)$.

2.9. Histochemical assays for ROS and lipid peroxidation

Eight lateral root tips and eight selected leaves from the first to the seventh expanded leaf were employed to histochemical assays. The three most representative images were shown.

2.9.1. Hydrogen peroxide

In situ detection of H_2O_2 was done following the protocol proposed by Thordal-Christensen et al. [45]. Detached leaves were vacuum infiltrated under dark conditions with 10 mM potassium phosphate buffer, 10 mM NaN_3 and 0.1% (w/v) 3,3′-diaminobenzidine (DAB), pH 7.8. Leaves were incubated overnight under dark conditions and then clarified with 0.15% (w/v) trichloroacetic acid in 4:1 (v/v) ethanol:choloroform for 48 h before being photographed.

2.9.2. Superoxide radical

In situ detection of superoxide radical ($O_2^{\bullet -}$) was done essentially as described by Jabs et al. [46]. Detached leaves were vacuum infiltrated with 10 mM potassium phosphate buffer pH 7.8, 10 mM NaN_3, 0.1% (w/v) nitro blue tetrazolium (NBT) and 0.05% (v/v) Tween 20. The detached, infiltrated and NBT-treated leaves were then maintained for 30 min under daylight conditions, prior to discoloration of the leaves using the same method described above for detection of H_2O_2.

2.9.3. Lipid peroxidation

Histochemical detection of lipid peroxidation was done with Schiff's reagent, which detects aldehydes that can be generated from lipid peroxides [47]. Roots and leaves were incubated in Schiff's reagent for 20 min and 60 min, respectively. Then roots were washed by immersing in distilled water until appearance of red/purple color for determination of aldehydes indicating lipid peroxidation. Leaves were incubated in Schiff's reagent for 60 min and then were bleached by immersing in boiling ethanol during 60 min.

2.10. Determination of NADPH and NADP$^+$ content

Roots and leaves selected as it was mentioned in Section 2.2 were frozen in liquid N_2 and ground in a mortar. Aliquots of the different homogenates were suspended in hot 0.1 N NaOH or 0.1 N HCl solutions (1/3, w/v). NADPH is stable in the alkaline solution, while NADP$^+$ is stable in the acid solution. The extracts were centrifuged at 12,000 × *g* for 5 min. The supernatants were used to quantify the nucleotides, according to the enzyme cycling method [48].

2.11. Determination of proline, TBARS and proteins

Proline was extracted from 100 mg of tissue with methanol–chloroform–water (12:5:1) and quantified according to Borsani et al. [49]. Lipid peroxides were detected as TBARS by measurement of malonaldehyde absorbance at 532 nm and concentration was determined using an extinction coefficient of 156 mM^{-1} cm^{-1} [50]. The proline and TBARS were determined in plant tissues obtained as described in Section 2.2. Four replicates of each sample were assayed.

Protein concentration was determined using the Bio-Rad protein assay using BSA as standard.

2.12. Statistical analysis

Analysis of variance was performed with data from at least three independent experiments, and means from the results from experiments were compared using Tukey's test at the $p \leq 0.05$ level.

3. Results

3.1. Phenotype of L. japonicus under water stress

Plants were subjected to water stress as described in Section 2, and the phenotype of 35-day-old plants grown under control conditions or exposed to water stress for 5 days is shown in Fig. 1(a). Water stress caused a marked loss of turgor in both stems and leaves and dehydration of roots was evident in stressed plants compared with control plants and confirmed by relative water content (RWC), Fig. 1(b). Proline content was also determined in roots and leaves as a marker of plant water stress. Free proline content was notably higher in roots (23-fold) than in leaves (10-fold) of plants subjected to water stress versus plants maintained under control conditions (Fig. 1(c)), indicating that water stress was well established.

3.2. Effect of water stress on the metabolism of ROS

To verify potential ROS damage in *L. japonicus* exposed to water stress, different approaches were used. Histochemical detection techniques performed in root and leaves showed an accumulation of superoxide radical, hydrogen peroxide and aldehydes derived from lipid peroxidation in these organs subjected to water stress conditions (Fig. 2). Roots did not accumulate significantly levels of superoxide radical or hydrogen peroxide as consequence of water stress. However, leaves showed an increase in both ROS, and in case of superoxide radical, this increase was more evident in main and secondary veins (Fig. 2). A histochemical method employing Schiff's reagent was used to detect aldehydes normally generated from lipid peroxides. With this method a red/purple color that developed on tissue corresponds to the presence of aldehyde derived from oxidized lipid. Thus, Fig. 2(c) shows that treatment of roots of stressed plants exhibited a relatively intense red color throughout

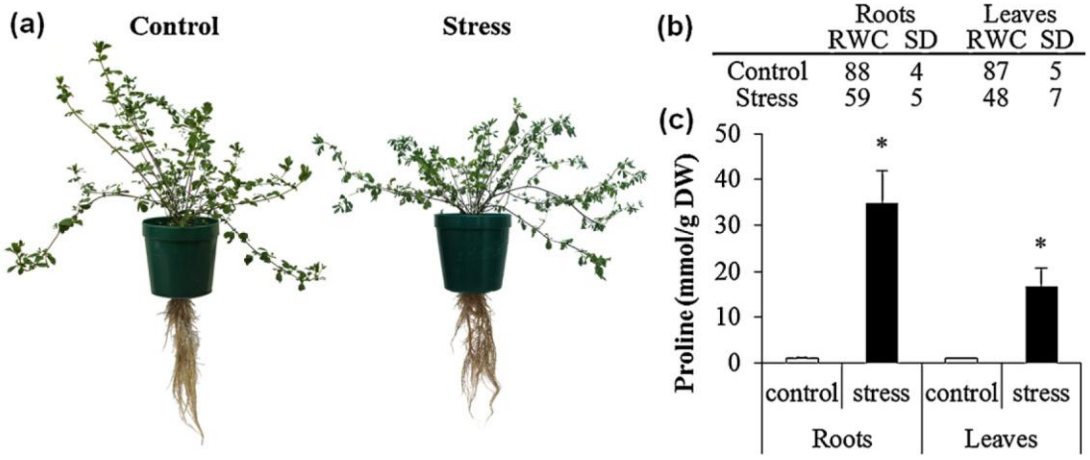

Fig. 1. Phenotype, relative water content (RCW) and proline accumulation of 35-day-old plants of *L. japonicus* exposed to water stress for 5 days. (a) Phenotype of 35-day-old plants exposed to water stress for 5 days, control plants (left) and water stress treatment (right). (b) Relative water content of roots and leaves in control and water stress condition. (a) Proline accumulation in plants subjected to water stress versus control conditions. Data are means ± SD of at least three biological replicates. Asterisks indicate that differences from control values were statistically significant at $p \leq 0.05$.

primary roots compared with controls. The Schiff's reagent assay in leaves showed a higher increase in the red/purple color compared with those obtained in roots (Fig. 2(c)), suggesting that the formation of aldehydes as consequence of water stress is higher in leaves than in roots. A quantitative analysis of lipid peroxidation measured as thiobarbituric reactive substances (TBARS) confirmed the damage in roots and leaves observed with Schiff's reagent. Fig. 3 shows a significant increase in the content of lipid peroxidation in stressed roots and leaves being 1.6 and 3.3-fold higher, respectively, compared with controls. These results are in agreement with the result presented in Fig. 2(c). As per these results, the increased lipid peroxidation in water stress plants suggested the existence of an oxidative stress.

Analysis of key antioxidant enzymes, including CAT, SOD and representative enzymes of the ascorbate–glutathione cycle was done to determine the extent of modulation of these enzymes

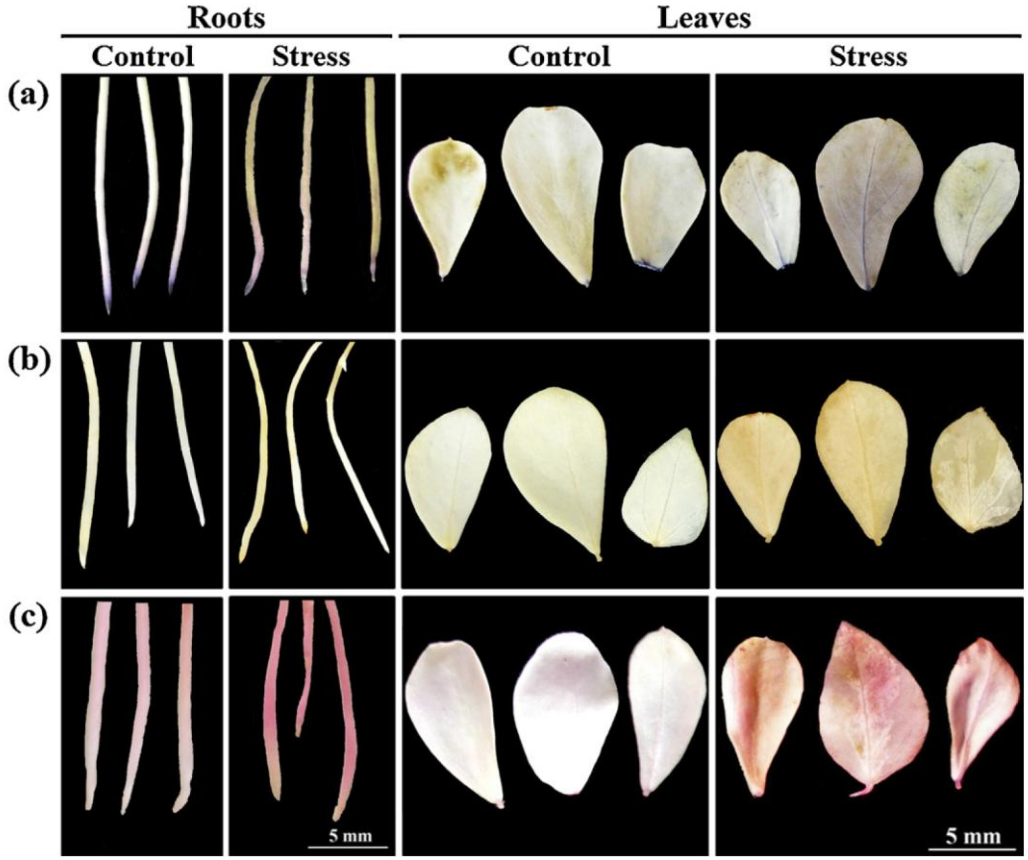

Fig. 2. ROS and lipid peroxidation in roots and leaves of 35-day-old plants subjected to water stress. (a) Superoxide in situ staining by NBT. (b) Hydrogen peroxide in situ staining by DAB. (c) Histochemical detection of aldehydes derived from lipid peroxidation. The red/purple color indicates the presence of lipid peroxidation detected with Schiff's reagent.

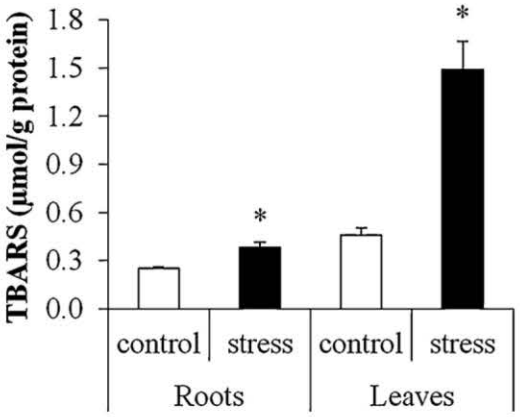

Fig. 3. Lipid peroxidation analysis of 35-day-old plants of *L. japonicus* exposed to water stress. TBARS concentration in roots and leaves. Data are means ± SD of at least three replicate measurements. Asterisks indicate that differences from control values were statistically significant at $p \leq 0.05$.

during water stress. Fig. 4(a) shows that during water stress CAT activity increased 1.7-fold in roots and 1.4-fold in leaves. Similarly, GR activity was increased 2-fold in both organs (Fig. 4(b)). In contrast, no significant changes were observed in APX activity in roots, however in leaves this activity increased 1.3-fold in response to water stress (Fig. 4(c)). Fig. 4(d) shows a native PAGE analysis of SOD activity in roots and leaves of *L. japonicus*. Based on the use of isozyme-specific inhibitors, one MnSOD, one FeSOD and two CuZn-SOD (I and II) were found in roots. In leaves, the same isozymes pattern were identified as previously reported for *L. japonicus* [51], with the exception of the FeSOD. Most root SOD isozymes showed a slight increase under water stress, with the exception of Fe-SOD that was not detected under this stress. No changes were observed in the activity of three SOD isozymes in leaves.

Table 1
NADPH and NADP$^+$ content in roots and leaves. NADPH and NADP$^+$ content are expressed as nmol g^{-1} DW. Data are the mean ± SD of at least three different experiments. *Differences from control values were significant at $p < 0.05$. DW, dry weight.

Plant organ	Metabolite	Control	Stress
Roots	NADPH	90.6 ± 7.2	27.3 ± 2.0*
	NADP$^+$	83.9 ± 5.4	18.6 ± 2.1*
	NADPH/NADP	1.08	1.47
Leaves	NADPH	23.3 ± 1.0	17.4 ± 1.1*
	NADP$^+$	30.3 ± 2.9	23.1 ± 1.3*
	NADPH/NADP	0.77	0.75

Considering that NADPH is required for the ascorbate–glutathione cycle which is also essential for maintenance of cellular redox state, activity of the main NADPH-generating enzymes was assayed including: G6PDH, 6PGDH, ME and ICDH. Fig. 5 shows that these activities were significantly affected in roots during water stress. Whereas activity of G6PDH and ICDH were reduced 6.5-fold and 1.5-fold, respectively (Fig. 5(a) and (c)), 6PGDH and ME increased 1.5-fold and 1.3-fold, respectively (Fig. 5(b) and (d)). In most cases, response of leaf enzymes to water stress was almost negligible in comparison with root enzymes. None of the leaf NADP-dehydrogenases seemed to be affected with the exception of G6PDH that was decreased ca. 50% under water stress (Fig. 5(a)). Additionally, the contents of NADPH and NADP$^+$ were also determined as good indicators of the redox state of cells. In general, the contents of NADPH and NADP$^+$ were significantly higher in roots than in leaves (Table 1) which is well correlated with the higher activity detected for the different NADP-dehydrogenase. Under stress conditions, the contents of NADPH and NADP$^+$ in roots were reduced 3.3-fold and 4.5-fold, respectively which provoked a rise in the NADPH/NADP$^+$ ratio (Table 1). However, the contents of NADPH and NADP$^+$ in leaves

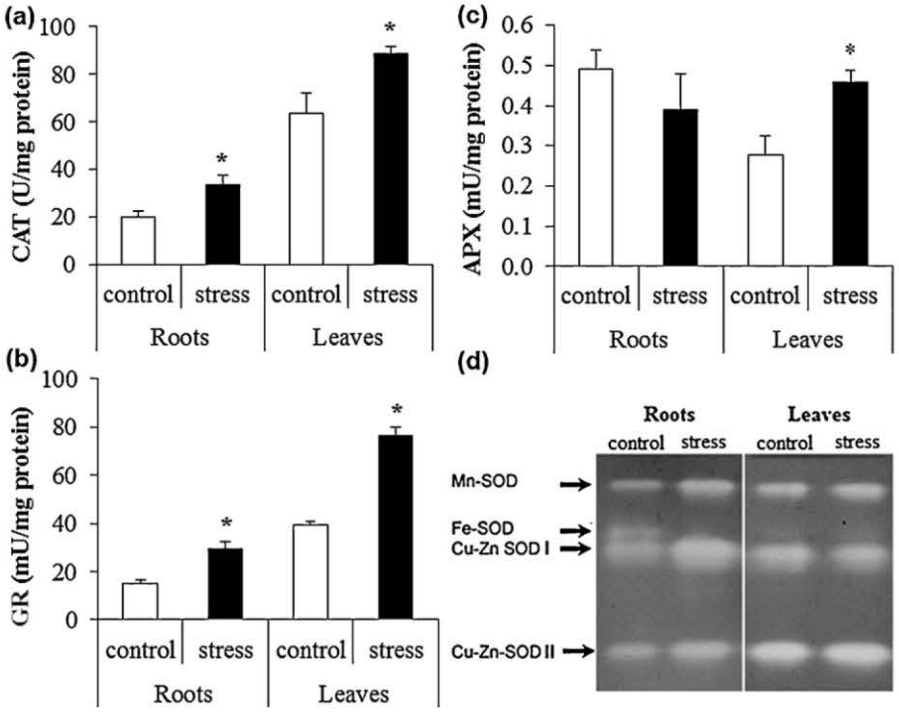

Fig. 4. Analysis of ROS metabolism in roots and leaves of 35-day-old plants subjected to water stress. (a) CAT activity, (b) GR activity, and (c) APX activity. Data are means ± SD of at least three biological replicates. Asterisks indicate that differences from control values were statistically significant at $p \leq 0.05$. (d) Analysis of SOD isozymes. SOD isozymes were separated by native-PAGE and stained by a photochemical method using 30 µg of protein per lane. The arrows indicate the SOD isozymes previously identified in leaves of *L. japonicus* by specific inhibitors [51].

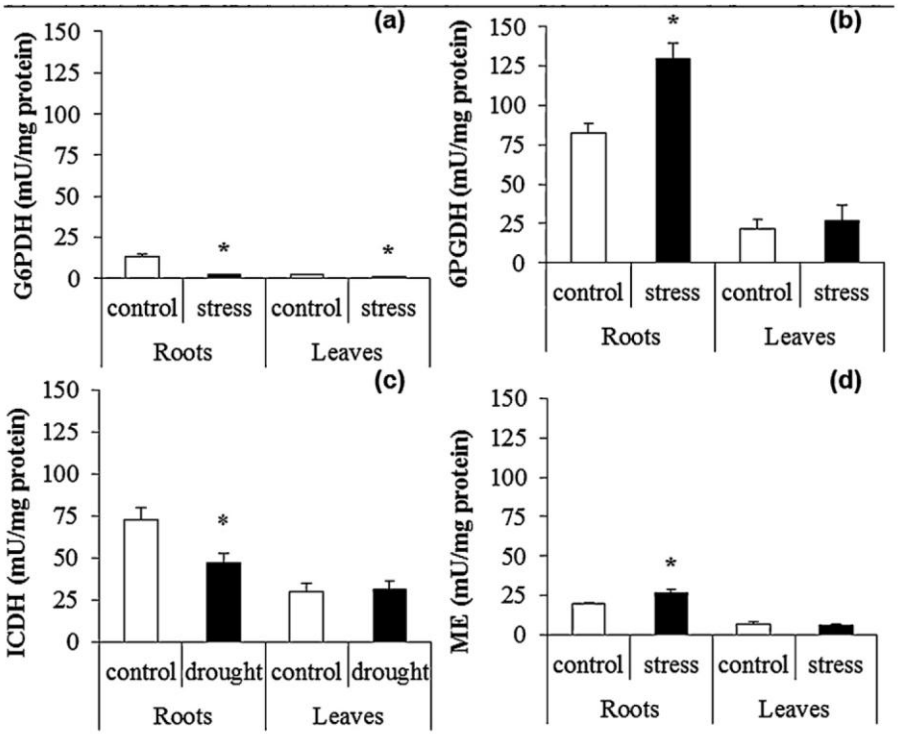

Fig. 5. Analysis of NADP-dehydrogenase activities in roots and leaves of 35-day-old plants subjected to water stress. (a) G6PDH, (b) 6PGDH, (c) NADP-ICDH, and (d) ME. Data are means of ±SD three biological replicates. Asterisks indicate that differences from control values were statistically significant at $p \leq 0.05$.

were 1.3-fold reduced for both nucleotides. Consequently, the NADPH/$NADP^+$ ratio was more affected in roots than in leaves.

3.3. Effect of water stress on the metabolism of RNS

Fig. 6(a) shows that NO content increased 1.8-fold in roots and 1.3-fold in leaves under water stress. Fig. 6(b) shows that whereas the GSNOR activity decreased 23% in roots, no significant changes were observed in leaves. Root NO content was considerably affected by water stress, and this was supported by visualization of NO localization in roots using confocal laser scanning microscopy with DAF-2 DA as fluorescent probe. Fig. 7(a) shows that the distribution of NO in a root tip of plants maintained under control conditions was distributed uniformly in the root. Likewise, under water stress NO was similarly distributed, but a large increase in root NO content was evident (Fig. 7(b)). Tyrosine nitration of protein is a well-established consequence of NO accumulation and thus a marker for nitrosative stress. Fig. 8 shows an immunoblot analysis of protein tyrosine nitration in roots and leaves of water stressed plants. A distinct protein nitration profile was found in roots and leaves. Moreover, there was a dramatic increase in nitration of at least three polypeptides of 41 kDa, 35 kDa and 28 kDa in roots of water stressed plants versus control plants, and no bands were observed under control conditions. Protein tyrosine nitration in leaves was evident in both water stressed and control plants. There was an increase in nitration of two immunoreactive bands of 53 kDa and 43 kDa in leaves of water stressed plants versus control plants, and the 43 kDa band was not detected in control plants.

4. Discussion

Water stress in plants remains one of the most significant factors affecting plant growth and productivity worldwide. Well-documented strategies for adaptation and recovery from conditions of water stress include: reduction in leaf size and water use, stomatal closure, and root proliferation [52–55]. Water stress is also well-known to be accompanied by oxidative stress, however, much less is known about the participation of RNS and NO metabolism. The main goal of this study was to analyze the potential relationship between RNS and ROS metabolism in roots and leaves under water stress and establish if this environmental stress is accompanied by a nitro-oxidative stress using specify markers such as: increased levels of proline, lipid peroxidation and protein tyrosine nitration.

4.1. Water stress induces an oxidative stress mainly in leaves

A general response of plant cells to water deficit is to accumulate low-molecular-mass compounds such as proline, glycine betaine, sugars and polyols in the cytoplasm to maintain ion balance in vacuoles [56]. In the case of proline, experimental data have shown that proline accumulation can be used as an indicator of water stress in *L. corniculatus* [57] and in *L. japonicus* [58]. In this study, leaves and roots of water stress-stressed plants showed significant increases in proline, particularly in roots. This amino acid is relevant in the mechanism of response against water stress and it has been suggested that proline is synthesized in the aerial part to regenerate $NADP^+$ and then is translocated to roots where the catabolism of proline is required for growth at low water potential [59]. However, proline seems to have additional roles including its ability to act as a molecular chaperone able to protect protein integrity as well as to enhance activities of specific enzymes [60,61]. Water stress also resulted in increased oxidative stress by ROS accumulation in both organs but more in leaves than in roots. Oxidative stress lead to cellular damage, in our experimental conditions both aldehyde and lipid peroxidation content, were markedly higher in stressed leaves compared with roots. Similar behavior of increased activity by water stress in leaves and roots were found for antioxidant enzymes such as CAT, GR and APX, however, higher activities and levels of induction were found in leaves compared with roots.

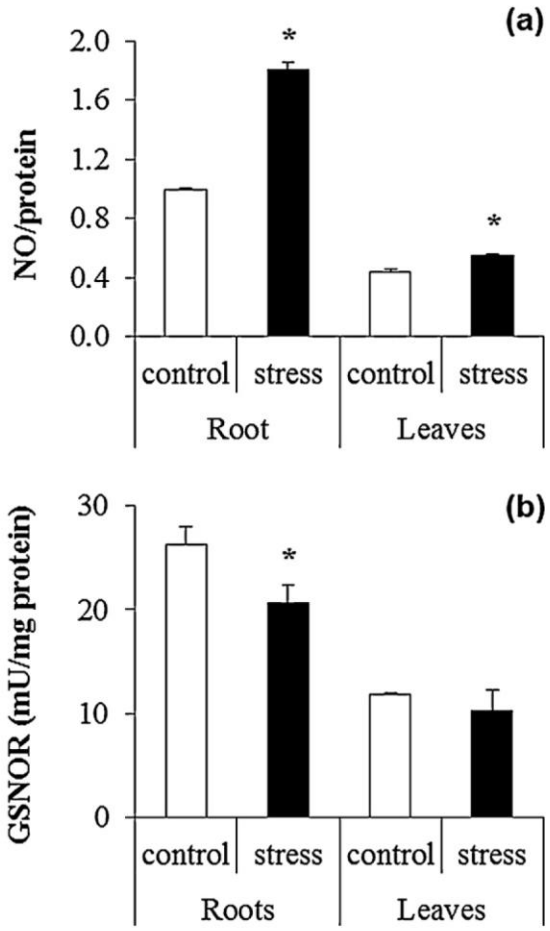

Fig. 6. Analysis of RNS metabolism in roots and leaves of 35-day-old plants subjected to water stress. (a) NO content determination by spectrofluorometric assay using DAF-2 as fluorescence probe. The fluorescence produced is expressed as arbitrary units relative to mg protein. (b) GSNOR activity. Data are means ± SD of at least three biological replicates. Asterisks indicate that differences from control values were statistically significant at $p \leq 0.05$.

SOD isozymes, in contrast, exhibited different patterns of activity in roots and leaves of water stress-stressed plants. Adaptation of roots under water stress resulted in complete down-regulation of FeSOD, whereas CuZnSOD increased significantly. Similar drought adaptation responses have been described for other plants, i.e. in *Medicago truncatula* water stress resulted in an increase in oxidative damage accompanied by an increase in gene expression of antioxidant enzymes including CAT, cytosolic APX, CuZnSOD, and FeSOD. A clear demonstration of adaptation was illustrated by this plant specie, since drought-induced oxidative damage responses were reversed following watering [62]. Progressive drought stress of *Vigna radiata* resulted in specific up regulation of CuZnSOD when analyzed by proteomic methods [63]. In the same way, analysis of detached maize leaves (*Zea mays*) treated with polyethylene glycol, which simulates drought stress, resulted in increased activity of chloroplastic and cytosolic anti-oxidant enzymes: SOD, APX, and GR [64].

NADPH is a key cofactor in cellular redox homeostasis and is also involved in detoxification processes [65,66]. Processes leading to regeneration of cellular NAPDH can therefore be considered potential secondary defense systems [13,67]. However, it should be also considered that an excessively high NADPH/$NADP^+$ ratio can cause a shortage of electron acceptors in the chloroplast and increased ROS production. Regarding enzyme activities that can modulate NAPDH levels, a different response was observed in the two organs where NADP-dehydrogenase activities were more affected in roots than in leaves under water stress in *L. japonicus*. Thus, whereas G6PDH and ICDH were found to be decreased in roots under water stress; 6PGDH and ME were induced. In leaves, however, only the G6PDH was slightly inhibited by water stress. Considering the total contribution of NADP-dehydrogenase activity of all enzymes evaluated we propose that while roots exhibit an increase of NADP-dehydrogenase activity, in leaves a slight decrease was observed. Although the contents of NADPH and $NADP^+$ were clearly affected under water stress in leaves and roots, it must be pointed out that the NADPH/$NADP^+$ ratio in root systems increased which is in agreement with the behavior of the NADP-dehydrogenase activities in this organ contributing to mitigation of the oxidative stress damages in comparison to leaves. In contrast with our study involving several NADP-dehydrogenases and two organs (leaves and roots) under a specific stress, previous studies have been usually focused in one organ or in one specific NADP-dehydrogenase.

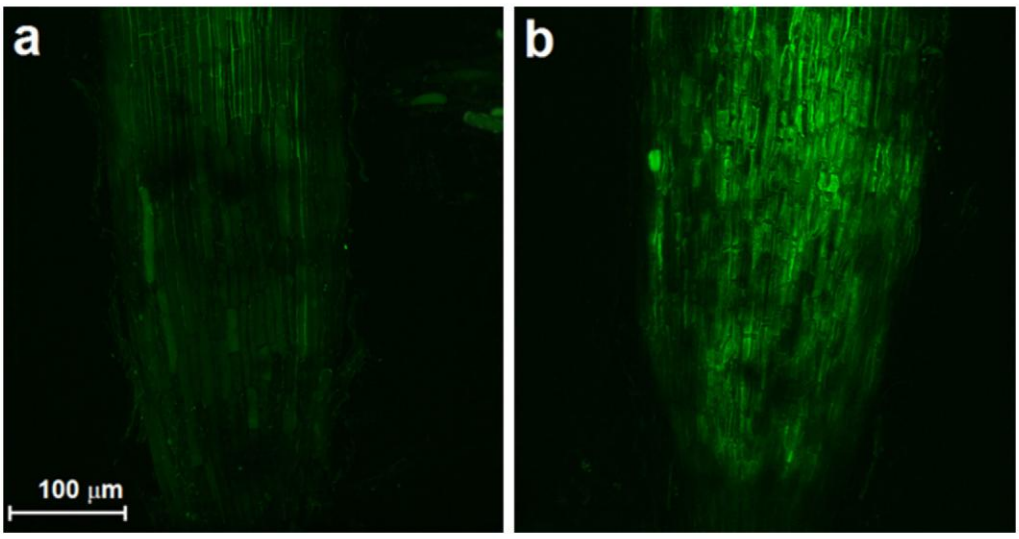

Fig. 7. Representative images illustrating CLSM detection of NO in apex of roots of 35-day-old plants subjected to water stress. The bright green fluorescence corresponds to the detection of NO in different longitudinal root sections using the fluorescence probe DAF-2 DA. (a) NO in roots of control plants. (b) NO in roots of plants under water stress. The images are representative of seven roots visualized.

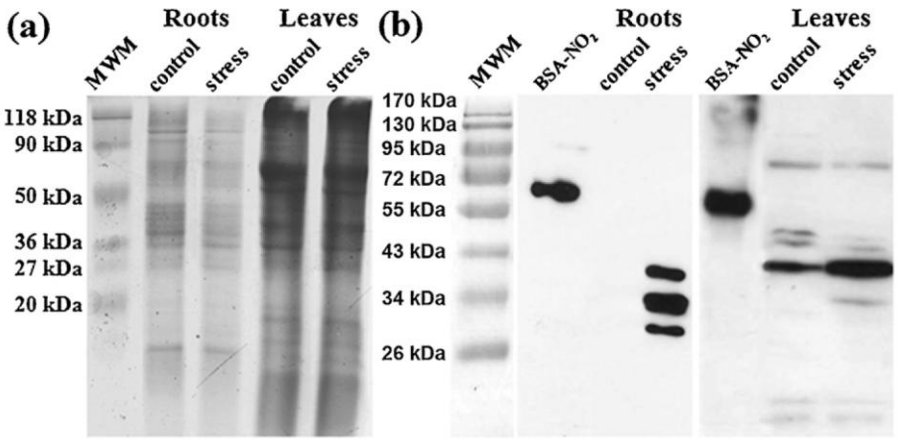

Fig. 8. Representative immunoblots showing protein tyrosine nitration in roots and leaves of 35-day-old plants subjected to water stress. Roots (20 μg protein per lane) and leaves (60 μg protein per lane) samples were separated by SDS-PAGE and analyzed on Western blots. (a) Coomassie staining. (b) Western blot anti-nitrotyrosine. Commercial nitrated BSA (NO_2-BSA) was used as a positive control.

For example, in leaves of olive plants grown with 200 mM NaCl which provokes an oxidative stress, a 30–50% increase in activity of all NADP-dehydrogenases was found [13]. *Arabidopsis* seedlings exposed to 100 mM NaCl induced oxidative stress, and analysis of NADP-dehydrogenases showed that with the exception of 6PGDH, other dehydrogenases were significantly increased in activity. ICDH had the highest relative specific activity (1.9-fold) under salinity conditions in that study [67]. Taken together, the antioxidant enzymes and NADP-dehydrogenase from roots and leaves showed a differential mechanism of response against water stress in leaves and roots being the leaves the organ apparently most affected for a process of oxidative stress.

4.2. Water stress provokes a nitrosative stress mainly in roots

Protein tyrosine nitration was increased by water stress in both roots and leaves, but was more pronounced in roots. Unlike leaves, in roots there was a strong induction in nitration of at least three proteins that were not detected under control conditions. This result possibly suggests that the root proteins either become more susceptible to nitration or nitration of these proteins plays a role in protection from nitro-oxidative stress. In this sense, an increase of protein nitration has been observed under different stress conditions which were accompanied by a nitrosative stress [22,29,68,69]. A recently work in citrus plants, showed also the presence of nitrated proteins in roots and leaves subjected to salt stress [70]. In the same work, they also studied the carbonylated and *S*-nitrosylated proteins, and interestingly they observed a higher numbers of carbonylated proteins in leaves than in roots, as consequence of the oxidative stress, and a more number of nitrated and nitrosylated proteins in roots than in leaves, as consequence of nitrosative stress [70]. As per the results, they also found that under salinity conditions, roots are more affected by a nitrosative stress whereas leaves are more affected by oxidative stress.

In addition, we showed that water stress results in an increase in NO content and a reduced GSNO reductase activity, especially in roots. The consequence is a rise of GSNO and nitrosothiols (SNOs) cellular contents that, under oxidative stress conditions could stimulate protein tyrosine nitration. Similar observations relating reduced GSNOR activity, increased SNOs levels and protein tyrosine nitration have been also described under other stress conditions such as high temperature and mechanical wounding in pea leaves and sunflower hypocotyls [22,29,68] or low temperature in pepper leaves [69]. On the other hand, there are only few reports in which NO metabolism has been studied in response to water stress and most of them are using sodium nitroprusside (SNP) as an exogenous source of NO. Thus, water stress resulted in an increase in water retention induced by stomatal closure of detached leaves of wheat seedlings pre-treated with 150 μM SNP [71]. In the same way, leaves of *Poncirus trifoliata* seedlings pre-treated with 100 μM SNP that were then subjected to water stress exhibited: less water loss, lower electrolyte leakage and ROS accumulation, higher antioxidant enzyme activity and smaller stomatal apertures compared with controls [72]. Water stress induces a simultaneous accumulation of •NO and proline in rice, but NO is not essential for water stress-induced proline accumulation in leaves. Foliar spray of SNP increases endogenous •NO content, induces stomatal closure, decreases transpiration rate and enhances the adaptive responses against water stress in rice [73]. Similar pharmacological approaches using an •NO donor, scavenger or an inhibitor of nitric oxide synthase activity in maize suggest that •NO is part of a signaling component in a mechanism of water stress tolerance in maize seedlings [74]. In addition, in *Cynodon dactylon*, ABA treatment improves water stress tolerance since ABA induces production of H_2O_2 and •NO to mediate enhanced antioxidant enzyme activities such as CAT and SOD. In that study it was suggested that •NO acts downstream of H_2O_2 [75].

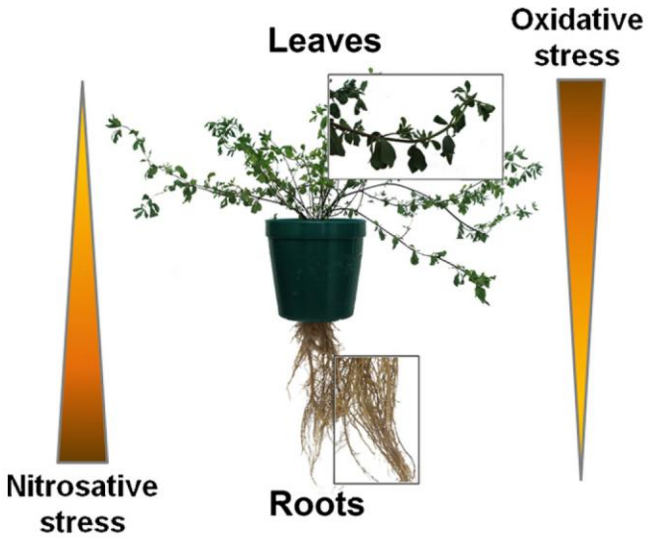

Fig. 9. Model for interactive oxidative and nitrosative components in response to water stress. Nitrosative stress is based in the increase of protein Tyr nitration and oxidative stress is based in the increased of TBARS.

In summary, it is proposed that water stress in *L. japonicus* causes nitro-oxidative stress which supports the increased lipid oxidation and protein Tyr nitration. A differential distribution of the severity of oxidative and nitrosative components accompanying water stress was observed whereas the oxidative stress was higher in leaves, and the nitrosative stress was higher in roots (Fig. 9). The results obtained by Tanou et al. in citrus plants subjected to salt stress [70] also support this idea. Therefore, the differential spatial distribution of oxidative and nitrosative stress opens new perspectives for understanding potential interconnections between these classes of reactive species that accompany water stress, where proline and •NO could act as signal molecules.

Acknowledgements

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Capítulo 2. La prolina en la protección frente al daño nitro-oxidativo

Debido a las limitaciones encontradas para evaluar la funcionalidad de la prolina mediante el uso de las líneas transgénicas, se recurrió a diferentes aproximaciones como el modelado químico y experimentos *in vitro*.

I. La prolina no extingue al oxígeno singulete

Con el fin de establecer si la prolina protege frente al oxígeno singulete ($^1O_2$) inactivándolo por *quenching* físico o reaccionando con éste por *quenching* químico, se evaluó si había consumo de O_2 al poner a reaccionar prolina con $^1O_2$, lo que indicaría que hay *quenching* químico. Esta determinación se hizo con un *Oxygraph* (Hansatech Instruments) que consiste en un electrodo de oxígeno polarográfico de tipo Clark. Al no observa consumo de oxígeno por esta técnica, se infería que se trataba de *quenching* físico. Sin embargo al hacer un ensayo de competitividad y ver que el la presencia de prolina no afectaba el consumo de oxígeno por un *quencher* químico, nuestros resultados sugerían que no había ningún tipo de *quenching*. Con técnicas más directas se logró verificar que la prolina no protege del $^1O_2$ como se creía. Adicionalmente se logró dar una explicación a los resultados que llevaron a una interpretación incorrecta.

Los resultados de este trabajo se presentan en el siguiente artículo (Signorelli et al., 2013*c*).

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Short communication

Proline does not quench singlet oxygen: Evidence to reconsider its protective role in plants

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ABSTRACT

Plants are commonly subjected to several environmental stresses that lead to an overproduction of reactive oxygen species (ROS). As plants accumulate proline in response to stress conditions, some authors have proposed that proline could act as a non-enzymatic antioxidant against ROS. One type of ROS aimed to be quenched by proline is singlet oxygen ($^1O_2$)—molecular oxygen in its lowest energy electronically excited state—constitutively generated in oxygenic, photosynthetic organisms. In this study we clearly prove that proline cannot quench $^1O_2$ in aqueous buffer, giving rise to a rethinking about the antioxidant role of proline against $^1O_2$.

1. Introduction

Plants are subjected to environmental stresses that bring together an overproduction of reactive oxygen species (ROS) responsible for cellular oxidative damage and (programmed) cell death [1]. Plants accumulate proline (Pro) in response to water stress conditions [2] and different hypotheses have been put forward to explain the role of Pro under this type of stress and others that also result in Pro accumulation. However, none of these hypotheses has been incontrovertibly demonstrated [3,4]. One hypothesis is that Pro could non-enzymatically react with ROS and prevent essential molecules from (photo)oxidative damage [5–7]. Smirnoff and Cumbes evidenced that Pro was an effective hydroxyl radical (•OH) scavenger [6] and a rate constant of 4.8×10^8 M^{-1} s^{-1} for this reaction was determined [8]. In addition, Matysik and co-workers (2002) [9] proposed a role of singlet oxygen ($^1O_2$) scavenger for Pro in plants, but the rate constant for the reaction of Pro with $^1O_2$ is still unknown. The $^1O_2$ is generated with high UV doses or visible light in the presence of photosensitizers *in vitro* (or chlorophylls in green tissues of plants) and its deactivation may occur by phosphorescence emission or radiationless processes including electronic-to-vibrational energy transfer, charge transfer and electronic energy transfer [10]. Alia and co-workers (2001) [7] proposed that Pro could deactivate $^1O_2$ by physical quenching or by forming products such as superoxide radical ($O_2^{\bullet-}$) or peroxide anion $[O{-}O]^{2-}$. Since then, the protective role of Pro against $^1O_2$ has been widely considered by the scientific community. Nevertheless, the study by Alia and co-workers (2001) [7] was performed in ethanol and did not unambiguously elucidate if Pro could act as a physical or chemical quencher. If Pro reacted with $^1O_2$ producing $O_2^{\bullet-}$ or $[O{-}O]^{2-}$, Pro should not be regarded as an efficient non-enzymatic antioxidant in plants because its reaction with $^1O_2$ yielded other types of ROS and, additionally, Pro would be rapidly depleted. However, if Pro could physically quench $^1O_2$, it would not be consumed nor would it yield any additional ROS.

Direct detection of the phosphorescence emission of $^1O_2$ at 1270 nm is nowadays the only technique that allows researchers to establish unambiguously whether $^1O_2$ is produced in their system under study or whether the presence of additional compounds can affect its phosphorescence quantum yield or temporal profile. Because the quantum yield of $^1O_2$ emission in an aqueous or biological system

Abbreviations: CAT, catalase; EPR, electron paramagnetic resonance; FFA, furfuryl alcohol; LED, light-emitting diode; NaPB, 20 mM sodium phosphate pH 7.2; MB, methylene blue; Pro, proline; ROS, reactive oxygen species; SOD, superoxide dismutase; TEMP, 2,2,6,6-tetramethylpiperidine; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl.

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is as low as 10^{-6}–10^{-7} and its lifetime rather short, from few microseconds to few hundreds nanoseconds [10,11], researchers engaged in $^1O_2$ detection use indirect methods to monitor $^1O_2$ production and quenching instead. In these latter cases, $^1O_2$ production and quenching is followed by monitoring the accumulation of a new product that reacts with $^1O_2$ or the consumption of molecular oxygen with spectrophotometric, spectrofluorometric, polarographic or EPR spin trapping techniques [7,12–14]. The combination of direct and indirect methods can be useful for a better understanding of this issue, particularly when other ROS can come into play.

In our attempt to elucidate the bimolecular rate constants for the quenching of $^1O_2$ by Pro in neutral aqueous solutions, we reach the conclusion that Pro is, in fact, an inefficient $^1O_2$ scavenger.

2. Results and discussion

2.1. Direct detection of $^1O_2$

The temporal profile of the phosphorescence emission by $^1O_2$ at 1270 nm produced by riboflavin in the presence and absence of Pro under oxygen atmosphere is depicted in Fig. 1. The observed kinetic traces were fit well to the biexponential function $I(t) = a(k_1 - k_2)^{-1}[\exp(-k_2t) - \exp(-k_1t)]$, where $a > 0$, and the larger and smaller of the two rate constants determine the rise and decay of the emission signal respectively; here k_1 is identified with the larger rate constant and *vice versa*. Before the analysis of the rate constants in 20 mM sodium phosphate pH 7.2 (NaPB), experiments with NaN_3 or under a stream of N_2 were performed to unambiguously correlate the origin of the kinetic traces with $^1O_2$ emission (data not shown). The temporal profile of $^1O_2$ emission showed that $\tau_1 \equiv 1/k_1 = 0.7$ μs. A value that agrees with the expected value under oxygen atmosphere [11], where the concentration of dissolved oxygen is found to be ~3.2 times larger [15,16]. The decay time constant $\tau_2 \equiv 1/k_2$ was slightly larger in 20 mM NaPB (4.5 μs) than that previously determined in pure water (3.7 μs) [15]. When Pro was added up to a concentration of 150 mM, mimicking the average natural accumulation of Pro in aqueous compartments of plant cells in response to adverse environmental stresses, neither the intensity of the $^1O_2$ emission nor the rise and decay constants changed within experimental error (Fig. 1), indicating that first Pro does not affect triplet–triplet energy transfer from riboflavin to molecular oxygen and second Pro does not efficiently deactivate $^1O_2$. Similar conclusions were reached when lumiflavin or toluidine blue in 20 mM NaPB or ethanol were used as $^1O_2$ photosensitizers instead of riboflavin (data not shown).

2.2. Oxygen consumption

The putative reaction of Pro with $^1O_2$ was also investigated indirectly in a Clark-type electrode, keeping a nearly constant concentration of $^1O_2$ while exciting methylene blue (MB) with a red light-emitting diode (LED) source. In this experiment, the formation and deactivation of $^1O_2$ and, presumably, other types of ROS were investigated. Fig. 2 shows that the addition of 100 mM Pro did not bring together any consumption of oxygen after the dark-to-light shift, indicating that $^1O_2$ photosensitized by MB is simply deactivated by H_2O molecules. In contrast, the addition of furfuryl alcohol (FFA)—a well-known chemical quencher of $^1O_2$ [17]—at a concentration of 0.4 μM consumed oxygen with a rate of 2.0 μM s^{-1}. The rate of oxygen uptake by FFA decreased to ~40% of its initial value when a physical quencher such as NaN_3 at a concentration of 1 mM was present. In contrast, the combined addition of 100 mM Pro and 0.4 μM FFA did not affect the oxygen uptake by FFA, confirming that Pro is both an inefficient physical and chemical quencher of $^1O_2$. Increasing the lifetime of $^1O_2$ by replacing H_2O by D_2O (*i.e.*, 20 mM potassium phosphate pD 7.2) did not enhance the quenching properties of Pro (data not shown). To establish unambiguously that the oxygen consumption observed in the Clark-type electrode was due to $^1O_2$ photosensitization, but not to the production of $O_2^{\bullet-}$ or H_2O_2, further experiments were performed in the presence of enzymatic antioxidants (*i.e.*, superoxide dismutase, SOD, and catalase, CAT). Fig. 2 shows that the addition of 500 U mL^{-1} of SOD or CAT did not induce any change in the photoinduced rate of oxygen uptake, even when 100 mM Pro was present in the medium, indicating that Pro does not react to produce $O_2^{\bullet-}$ or $[O{-}O]^{2-}$ either.

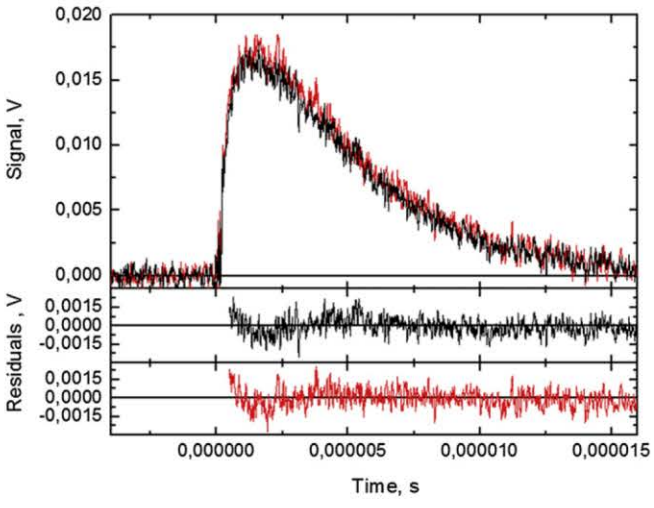

Fig. 1. Temporal profile of $^1O_2$ phosphorescence in the presence and absence of Pro. $^1O_2$ was photosensitized by riboflavin in 20 mM NaPB with no further additions (black traces) and 90 mM Pro (red traces) under oxygen atmosphere. The absorbance of riboflavin was 0.9 at 445 nm. The phosphorescence emission was measured at 1270 nm. The number of averaged scans was 128. The residuals are also shown for both experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

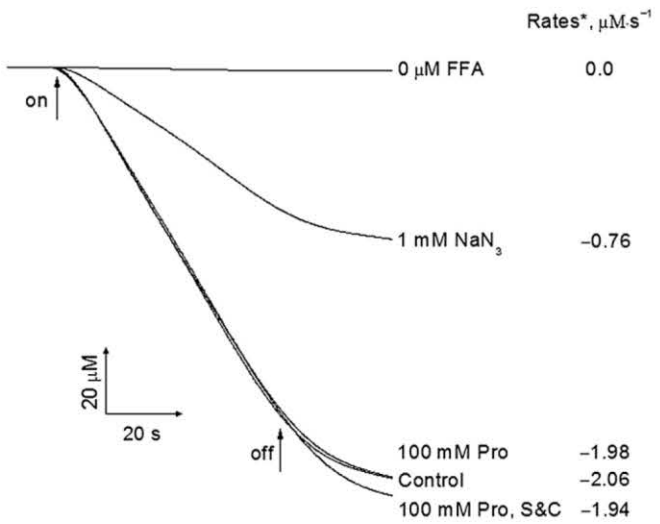

Fig. 2. Oxygen uptake in the Clark-type electrode chamber using MB as $^1O_2$ photosensitizer, and Pro and FFA as $^1O_2$ quenchers. Experiments were carried out in 20 mM NaPB. FFA at a concentration of 0.4 μM was present in all the experiments except when indicated. The absorbance of MB was 0.15 at 665 nm. S&C represents 500 U mL^{-1} of SOD and CAT. Arrows with on and off indicate when the red LED source was turned on and off respectively. * SD = ±0.06.

2.3. Deactivation by electronic-to-vibrational energy transfer

The results presented above show that the encounter of Pro with $^1O_2$ does not follow any process where an exciplex with partial charge transfer character becomes deactivated by intersystem crossing (physical quenching) or chemical reaction. To explore whether Pro could compete with H_2O for the deactivation of $^1O_2$ by electronic-to-vibrational energy transfer, the energy of the highest frequency mode vibration of Pro was compared with that of H_2O in liquid state. The water molecules in liquid state have stretch vibrations in the 3100–3600 cm^{-1} range and this vibration band has associated a rate constant for $^1O_2$ deactivation of ~2900 M^{-1} s^{-1} [10]. The highest frequency mode vibration (ν_{NH}) of Pro in water is 3057 cm^{-1} [18] and has a rate constant for $^1O_2$ deactivation of ~1530 M^{-1} s^{-1} [10], approximately half of the value for the stretching of water. These values show that Pro cannot efficiently compete with H_2O for $^1O_2$ deactivation through electronic-to-vibrational energy transfer in (cellular) aqueous media, based simply on the above values for the rate constants and the remarkable difference in concentration between the solute and the solvent.

2.4. Effect of Pro on TEMPO accumulation

The lines of evidence presented above prove that Pro cannot quench $^1O_2$. To better understand why our results differed from those observed by Alia and co-workers (2001) [7], $^1O_2$ production by toluidine blue was followed by electron paramagnetic resonance (EPR) spectroscopy under continuous illumination of the sample in the EPR cavity. The oxidation product of 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) grew continuously with the course of time in the absence of Pro; however, TEMPO accumulated slower in the first instance and turned down after several minutes in the presence of 25 mM Pro (Fig. 3). Additionally, the bandwidth of the EPR signal became narrower. This suggests that Pro do not prevent the oxidation of 2,2,6,6-tetramethylpiperidine (TEMP) by $^1O_2$, but the increase of TEMPO, indicating that the spin adduct with $^1O_2$ undergoes a change.

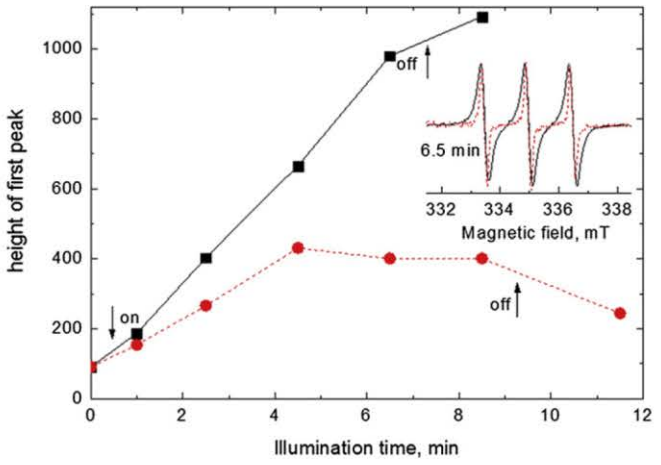

Fig. 3. Effect of Pro on TEMPO accumulation. The EPR signal was monitored in the absence of Pro (black squares and solid line) and 25 mM Pro (red circles and dotted line). A reaction mixture containing 1 mM toluidine blue and 10 mM TEMP was continuously irradiated with a 50 W Tungsten lamp with an optical fiber connected to the cavity (0.3 mW cm^{-2}) and the EPR signal of TEMPO monitored at different intervals. The inset shows the normalized EPR signal in the presence and absence of Pro for the comparison of the EPR bandwidth. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.5. Possible role of Pro in plants under stress

Pro accumulates in cytosol and chloroplast stroma [19]; where isoforms of the Δ-1-pyrroline-5-carboxylate synthetase enzyme are localized [20]. Taking into account our results, together with the fact that Pro accumulates in aqueous compartments of cells, but not in thylakoid membranes, where $^1O_2$ is constitutively produced by the photosystem II and largely damages lipids and membrane proteins under photoinhibition before diffusing into the surrounding medium, we propose that the Pro does not play any significant role in the scavenging of $^1O_2$ in plants. Several studies have concluded that oxidative damage is lower when Pro accumulates in plants under stress [21], but this protective effect must then be related to the scavenging of other ROS, for example •OH [6], but not $^1O_2$. In addition, Pro can play other protective roles; it can act as a compatible osmolyte [22,23] or the Pro synthesis and catabolism can play essential role in redox balance [24]. Probably, both Pro itself and Pro synthesis and catabolism play combined functions in the stress adaptation of plants.

3. Conclusions

In brief, we determine that Pro does not quench $^1O_2$ either chemically or physically using methods that include the direct detection of the $^1O_2$ emission, oxygen consumption in a Clark-type electrode or EPR spin trapping. Moreover, stretching vibrations of Pro do not exceed the water vibrations and so the presence of Pro in the medium does not increase the ability to quench $^1O_2$ by electronic-to-vibrational energy transfer. On the basis of these facts, we conclude that Pro accumulation does not play any significant role in the scavenging of $^1O_2$ in plants under stress and that other roles in osmoprotection, •OH scavenging or contribution in redox homeostasis should be considered.

4. Methods

4.1. Time-resolved $^1O_2$ measurements

Time-resolved emission of $^1O_2$ at 1270 nm was studied in 20 mM NaPB under oxygen atmosphere at room temperature. Further details about the experimental setup have already been described in sufficient detail in Arellano et al. (2007) and Li et al. (2012). An absorbance of 0.9 at 445 nm for riboflavin was used to photosensitize $^1O_2$ in the assay buffer. The concentration of Pro ranged from 0 to 150 mM.

4.2. Oxygen consumption

Oxygen uptake by $^1O_2$ scavengers was measured polarographically using a Chlorolab 2 system (Hansatech Instruments, England) at 20 °C. Samples were buffered at pH 7.2 with 20 mM NaPB and contained MB with an absorbance of 0.15 at 665 nm to photosensitize $^1O_2$. When needed, 0.4 μM FFA or 500 U mL^{-1} of SOD or CAT were added to scavenge $^1O_2$, $O_2^{\bullet-}$ or H_2O_2. All samples were incubated in the dark for 1 min before the red LED source was switched on. The light irradiance in the electrode chamber was 2 mE m^{-2} s^{-1}.

4.3. $^1O_2$ detection by spin trapping EPR

A reaction mixture containing 1 mM toluidine blue and 10 mM TEMP was continuously irradiated with a 50 W Tungsten lamp with an optical fiber connected to the cavity (0.3 mW cm^{-2}) and the EPR signal of TEMPO monitored at different intervals, in the absence or presence of 25 mM Pro. EPR measurements were done using a JEOL free radical monitor machine (JES-FR30) with a cylindrical cavity (TE011 mode) working in the 9.1–9.5 GHz range. The receiver gain of the EPR instrument was 200, modulation width 0.2 mT and

power 4 mW. Experiments were done at room temperature and in ethanol as a solvent.

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II. Mecanismos de reacción entre la prolina y el radical hidroxilo

Debido a la alta reactividad del radical hidroxilo (·OH), realizamos estudios *in silico* a nivel cuántico para establecer los mecanismos de reacción entre este radical y la prolina y determinar los posibles derivados estables. Para ello se consideraron los ataques del ·OH sobre los átomos de C del anillo de la prolina, ya que en distintos trabajos se propone que la reacción genera hidroxiprolina.

En esta sección se presenta un artículo (Signorelli et al., 2014) que demuestra que el ·OH reacciona con la prolina, principalmente por abstracción de H formando agua y produciendo prolina radical. Las diferentes abstracciones tienen barreras energéticas muy bajas, lo que concuerda con la rápida constante de reacción determinada de 4,8 x 10^8 $dm^3 \cdot moL^{-1} \cdot s^{-1}$ (Davies, 2005). Por otra parte los resultados demuestran que el ·OH ataca a la prolina preferentemente por la cara del grupo carboxilo. La prolina radical puede reaccionar con otro ·OH, esta posibilidad fue evaluada para probar la factibilidad de la formación de hidroxiprolina. Nuestro trabajo concluye que efectivamente la producción de hidroxiprolina puede darse por la adición directa del ·OH y la prolina radical. También demostramos que una segunda abstracción de H puede ocurrir en presencia de ·OH, y la barrera más baja fue encontrada cuando ocurre sobre el grupo NH_2^+. Esta abstracción puede llevar a la formación de P5C el cual es convertido enzimáticamente a prolina. Esto nos llevó a proponer un ciclo Pro-Pro donde una prolina reacciona con 2 ·OH y se transforma nuevamente en prolina con consumo de NADPH por la enzima P5CR.

Si bien el ·OH puede reaccionar con un amplio espectro de moléculas, la co-localización en el cloroplasto de éste con la prolina, la que alcanza la mayor concentración en dicho organelo (Büssis and Heineke, 1998), favorece el encuentro entre ambos y así las reacciones propuestas.

El ciclo Pro-Pro apoya a dos de los roles que se le han asignado a la prolina, el proteger frente a ROS y el de amortiguador redox por consumo de NADPH en tejido fotosintético. De este modo nuestra evidencia acompaña a la propuesta actual sobre las múltiples funciones de la prolina.

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Molecular Mechanisms for the Reaction Between •OH Radicals and Proline: Insights on the Role as Reactive Oxygen Species Scavenger in Plant Stress

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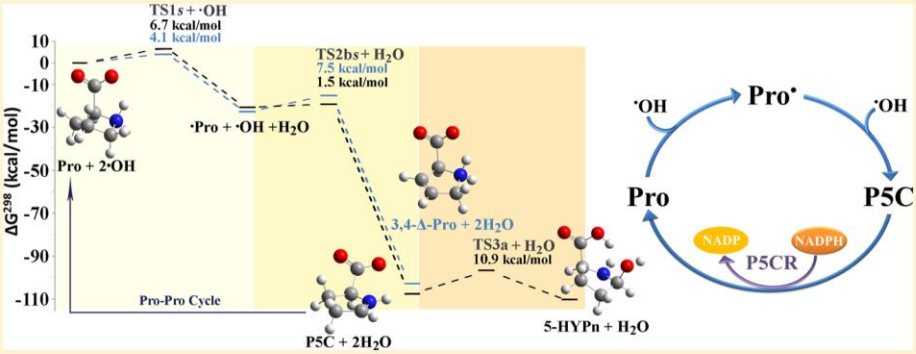
Supporting Information



ABSTRACT: The accumulation of proline (**Pro**) and overproduction of reactive oxygen species (ROS) by plants exposed to stress is well-documented. In vitro assays show that enzyme inactivation by hydroxyl radicals (•OH) can be avoided in the presence of **Pro**, suggesting this amino acid might act as a •OH scavenger. Although production of hydroxyproline (**Hyp**) has been hypothesized in connection with such antioxidant activity, no evidence on the detailed mechanism of scavenging has been reported. To elucidate whether and how **Hyp** might be produced, we used density functional theory calculations coupled to a polarizable continuum model to explore 27 reaction channels including H-abstraction by •OH and •OH/H_2O addition. The structure and energetics of stable species and transition states for each reaction channel were characterized at the PCM-(U)M06/6-31G(d,p) level in aqueous solution. Evidence is found for a main pathway in which **Pro** scavenges •OH by successive H-abstractions ($\Delta G^{\ddagger,298}$ = 4.1 and 7.5 kcal mol^{-1}) to yield **3,4-Δ-Pro**. A companion pathway with low barriers yielding Δ^1-pyrroline-5-carboxylate (**P5C**) is also supported, linking with **5-Hyp** through hydration. However, this connection remains unlikely in stressed plants because **P5C** would be efficiently recycled to **Pro** (contributing to its accumulation) by **P5C** reductase, hypothesis coined here as the "Pro-Pro cycle".

1. INTRODUCTION

When plants are exposed to biotic and/or abiotic stress, damage on cellular components (proteins, lipids, carbohydrates, and DNA) is induced as a result of the overproduction of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$), and hydroxyl radicals (•OH).[1−3] The latter is the most reactive species among ROS and can be generated in vivo both by Fenton's reaction or through homolysis of H_2O_2 under UV radiation, to which plants are highly exposed. Hydrogen abstraction, addition, and electron-transfer processes are the most common reaction channels for •OH, leading to new radicals or closed-shell species with lower reactivity.[4] Cellular defense against ROS involves both enzymatic and nonenzymatic antioxidant systems.[1−3,5] Superoxide dismutase, catalase, and peroxidase are representative enzymes whose induction is associated to stress acclimation.[5] Glutathione, ascorbic acid, and tocopherols are common examples of nonenzymatic systems involved in the antioxidant plant's defense,[1] a category to which some authors also ascribe proline.[6]

Accumulation of proline up to 100 times the normal level in stressed plants has been a well-known fact for more than 40 years,[7] reaching cytosol concentrations of 120−230 mM (see Ashraf and Foolad[8] and references therein). Accumulation by de novo synthesis observed under drought and high salinity conditions, UV/vis irradiation, oxidative stress, in presence of heavy metals, or as a response to different kinds of biotic stresses[9]

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has been reported to be a feature shared by a wide variety of organisms including bacteria, fungi, and plants.[10] Whereas some authors have proposed that proline could act as a compatible osmolyte,[11,12] others hypothesized it might act as a non-enzymatic antioxidant[6] maintaining the redox potential in the cell[13] and scavenging ROS such as $^{\bullet}OH$[14,15] and singlet oxygen ($^1O_2$)[16] (albeit for the latter some of us recently showed proline does not quench singlet oxygen[17]). Other authors also proposed alternative roles for the species, such as stabilizing protein structure[18] or acting as C/N storage.[19] Early in 1989, Sminorff and Cumbes showed that enzyme inactivation by $^{\bullet}OH$ can be avoided in vitro in the presence of **Pro**, proposing the molecule might act as a $^{\bullet}OH$ scavenger.[14] In vivo assays conducted by Jain et al. on callus subjected to saline stress showed its accumulation decreases lipid peroxidation, suggesting that proline might contribute to the antioxidant defense.[20] Moreover, indirect evidence also emerges in comparing oxidative damage under stress on genetically engineered plants containing transgenes for production of proline; whereas transgenics with increased accumulation under saline conditions suffer less oxidative damage,[21,22] those unable to accumulate proline exhibit a significantly lower tolerance to stress compared to wild-type plants.[23,24]

Although the concept of proline acting as an antioxidant has been frequently referenced during the last 20 years (more than 600 citations of the original article by Smirnoff and Cumbes[14]), proposals on a detailed mechanism of its reaction with $^{\bullet}OH$ and the physicochemical features of the species involved along the possible reaction paths are still lacking. The absence of experimental evidence is possibly due to the high reactivity of the radical, a feature that makes use of experimental approaches difficult. The only suggestion currently advanced is that proline could react with $^{\bullet}OH$ mainly forming 5-hydroxyproline (**5-Hyp**),[15] but no clear evidence of the presence of this molecule under plant stress has been reported yet. In this scenario, computational modeling by quantum methods such as those based on the density functional theory (DFT) appears as a very valuable approach to explore the features of possible pathways with atomic and electronic levels of detail.

Ab initio post-Hartree−Fock and DFT calculations have already been applied with success to characterize structural features of the most stable isomers both for neutral proline in vacuo[25−29] and for zwitterionic/ionized proline in solution.[30−35] Four stable isomers displaying internal hydrogen bond (HB) between the −C(=O)OH and −N(H) moieties have been found in the gas phase, resulting from the combination of two possible N···H−O/N−H···O=C HB patterns with the C^{γ} *endo*- and *exo*-like puckering conformations relative to −COOH.[29] The main species in aqueous solution at near-physiological pH corresponds to the zwitterionic form. According to DFT/PCM calculations[31] the structure also displays an internal $CO_2^{-}\cdots H-NH^{+}$ interaction with a HB distance of 1.85 Å (1.91 Å in a second ring-puckered conformer) which is lengthened by 0.05 Å when three explicit waters are included in the model. These findings are consistent with neutron scattering and Raman experiments pointing out hydration favors a transition from neutral to zwitterionic species through strong interactions with water molecules.[36] FTIR data suggests there are at most three HBs per molecule, with no evidence for extended HB networking.[37] Two ring-puckering down/up conformations of the zwitterion, respectively corresponding to C^{γ} and the C=O group lying on the same/opposite side of the $C^{\alpha}-N-C^{\delta}$ plane were also found to be shallow minima on the molecular potential energy surface (PES). A barrier of 1.72−2.08 kcal mol^{-1} (hysteretic dependence of energy on the angle was found) for interconverting them via ring twists was reported at the B3LYP/6-311++G** COSMO-(H_2O) level by Bouř and co-workers,[33] suggesting both conformers would be present under physiological conditions. Approximately equal Boltzmann populations are predicted at 298−300 K and pH 6.8−7.2 from several NMR-based analyses and computational modeling, with the down (C^{γ} *endo*) conformer slightly preferred in terms of energy (<1 kcal mol^{-1}).[32−34,38] Ionization state, conformational preferences, and HB are all elements that must be considered in properly describing how conditions resembling those in the cell of a stressed plant may modulate the reactivity of the amino acid and the susceptibility of each of the target sites toward radical attack (i.e., captodative effects[39] stabilizing C^{α} radicals are not present in a zwitterion).

Despite a considerable body of information has been built on studies of the primary steps for the reaction of $^{\bullet}OH$ with amino acids in peptides and proteins,[40−53] quite less is known at a detailed level for the H-abstraction from *free* amino acids, with a series of studies available in gas phase[54−57] and in aqueous solution.[58−64] These reactions are fast, in the near-diffusion limit, with an overall rate constant of 6.5×10^8 M^{-1} s^{-1} determined for proline in aqueous solution at pH 6.8 and 298.15 K.[58] The fate of the process over free amino acids is determined by the strength of the C−H bonds targeted by $^{\bullet}OH$ and the stability of the radicals thus produced as the main controlling factors[42] combined with the outcome of a subtle balance among attractive and repulsive interactions the approaching hydroxyl radical establishes with the carboxylate moiety.[47,49,65] Any possible H-atom abstraction is expected to be strongly exergonic, because the O−H bond dissociation energy (BDE) in H_2O (ca. 119.3−118.8 kcal mol^{-1})[66,67] is higher than the BDEs for any C−H bond in proline zwitterion. Moore and Julian[68] coupled DFT calculations with isodesmic reactions to evaluate on an equal footing BDEs for X−H bonds (X = C, N, O, S) along the 20 common amino acids as being in a peptide backbone. Values of 84.3, 98.7, 99.1, and 93.9 ± 2.4 kcal mol^{-1} were found for the C−H homolytic cleavage at the α, β, γ, and δ sites, respectively, in *N*-acetylproline amide. A significant increase in the C^{α}−H BDE is expected for free proline in the zwitterionic form, as replacement at the N-terminus of the acetyl cap by an amine and its protonation are opposite effects that globally destabilize radicals both at the backbone C^{α} and (to a smaller extent) at the adjacent side-chain carbon. Assuming transferability across amino acids for the overall magnitude of these effects (+12.8 kcal mol^{-1} for alanine[68]), a final value of 97.1 kcal mol^{-1} might in principle be expected for the C^{α} position in free proline, leading to the following ordering of C−H BDEs: δ (C5) < α (C2) < β (C3) ≅ γ (C4), following now the numbering shown in Scheme I. However, only side-chain radicals were found in an early ESR characterization of the spin-trapped free radicals produced by reaction of $^{\bullet}OH$ with proline in aqueous solution.[60] From that study, Rustgi et al.[60] inferred that (as other authors have also done[40,42]) the short-lived species with the unpaired electron at C5 would be the most stable product for the primary step of the reaction. Although some controversies are still open on the relative reactivity toward H-atom abstraction at each site of the side-chain,[61] those first findings excluding C2 proline radicals as an outcome of the reaction with $^{\bullet}OH$ were further confirmed by more recent mass spectrometry[69] and $^2H/^1H$ NMR[47,61] studies. On the basis of some of that evidence, Matysik et al. hypothesized that **5-Hyp** could be the main product of the $^{\bullet}OH$ scavenging activity of proline.[15] Here we

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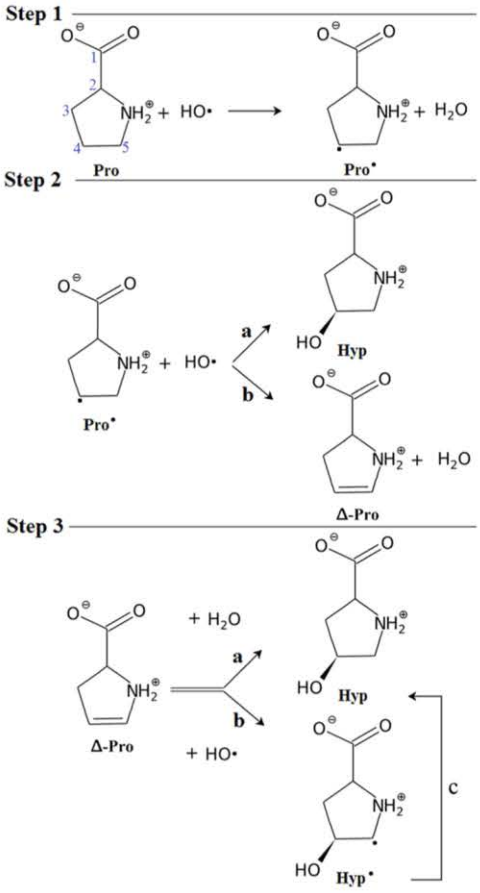
Scheme I. Possible Reaction Pathways Explored in the Attempt to Explain Formation of Hyp through •OH Scavenging by the Zwitterionic Species in Aqueous Solution[a]



[a]Atom numbering scheme is depicted over **Pro** in blue characters.

intend to shed light on these controversial aspects relying on computational models capable to find evidence, if any, in support for such scavenger activity by exploring the features of feasible reaction channels connected with the formation of hydroxyproline.

2. MATERIAL MODELS AND COMPUTATIONAL METHODS

2.1. Molecular Systems. Several reaction pathways starting with H-atom abstractions by •OH from the most stable conformer of the zwitterionic form of proline in aqueous solution (**Pro** hereafter) were extensively explored. A comparison with the features of the neutral species displaying an internal HB as conducted at the same level of theory is included as Figure S1 in the Supporting Information.

As depicted in Scheme I, the primary step in the reaction of •OH with **Pro** (identified as Step 1) was characterized for all the possible H-atom abstractions by radical attack from both faces of the pyrrolidine ring (i.e., from the side of the carboxylate group or from the opposite one, respectively labeled s and o channels) at C3, C4, and C5 in the side-chain as well as at C2 in the backbone. Two competitive channels of reaction starting from each of the stable carbon-centered radicals (**Pro•**) produced through Step 1 were further explored: the direct addition of •OH to generate **Hyp** (Step 2a) and a second H-atom abstraction from **Pro•** at the different alkyl sites adjacent to each center bearing the unpaired spin (Step 2b) leading to dehydroproline stable species (**Δ-Pro** in Scheme I) previously identified as intermediates in the H-abstraction of **Pro** by radicals, Δ^1-pyrroline-5-carboxylate (**P5C**) in particular.[42,69,70] Once again s and o labeling is used to distinguish among •OH attack to **Pro•** approaching the pyrrolidine ring from the same and opposite side of the carboxylate group, respectively. In closure of a mechanism capable of explaining hydroxyproline formation from **Δ-Pro** intermediates, two final competitive addition processes on the unsaturated C=C moieties were examined as follows: unassisted and assisted water addition eventually leading to production of **Hyp** (Step 3a) and unassisted and assisted •OH addition on the same target sites (Step 3b) eventually leading to distinct **Hyp•** radicals that finally would be able to generate **Hyp** by H-atom reabstraction (Step 3c) from protective species present in the cellular environment such as ascorbate, glutathione, α-tocopherol, carotenoids, and flavonoids.

2.2. Level of Theory. The geometrical structure of each stable species (reactants, products, and prereactive intermediate complexes IC) and transition states (TS) was fully optimized in aqueous solution at the (U)M06/6-31G(d,p) level[71] integrated with the IEF-PCM polarizable continuum model[72−74] without imposing symmetry restrictions and using solute cavities adapted to the molecular shape and constructed with Bondi radii.[75] An ultrafine pruned grid having 99 radial shells and 590 angular points per shell was employed for numerical integration in all M06 calculations to ensure small errors in reaction energies and barriers.[76] Expectation values of the spin operator S^2 were checked to be below 0.76/2.01 for all the open-shell species to ensure minimal spin contamination. The nature of each stationary point was carefully verified by inspection of the eigenvalues of the analytic Hessian in aqueous solution. Thermochemistry was evaluated at 298.15 K relying on the standard treatment for assessing thermal contributions (rigid rotor, harmonic vibrations with no scale factor, etc.) as implemented in the Gaussian09 program.[77] Nonelectrostatic contributions (cavitation, dispersion, and repulsion)[78,79] to the solvent free energy were also evaluated at 298 K. The reaction coordinate associated to each transition state (TS) was inspected by animation of the eigenvector associated to the imaginary frequency. Intrinsic reaction coordinate (IRC) minimum energy reaction paths[80] toward reactants and products were generated with the Hessian-based predictor-corrector (HPC) algorithm[81] including 45 steps for each side with a step size of 2 bohr amu$^{-1/2}$. Representative structures obtained from each side of the reaction path were thus used as the starting point for optimizing the structure of the corresponding intermediate complexes (ICs). UCCSD/cc-pVDZ geometry optimization in aqueous solution was also performed for the species involved in Step 3b which exhibited significant spin contamination at the DFT level. All the calculations were performed using Gaussian09, revision A.1.[77]

3. RESULTS AND DISCUSSION

3.1. First H-Atom Abstraction from Proline by ·OH: Regioselectivity of the Initial Attack. Figure 1 collects the structural features and free energy profiles in aqueous solution at 298.15 K for reactants, TSs, and products for six of the seven reaction channels explored for Step 1, including attack at C3, C4, and C5 from both sides of the pyrrolidine ring (s and o channels). H-abstraction from C2 (not shown) was also characterized for the only possible o-face of attack.

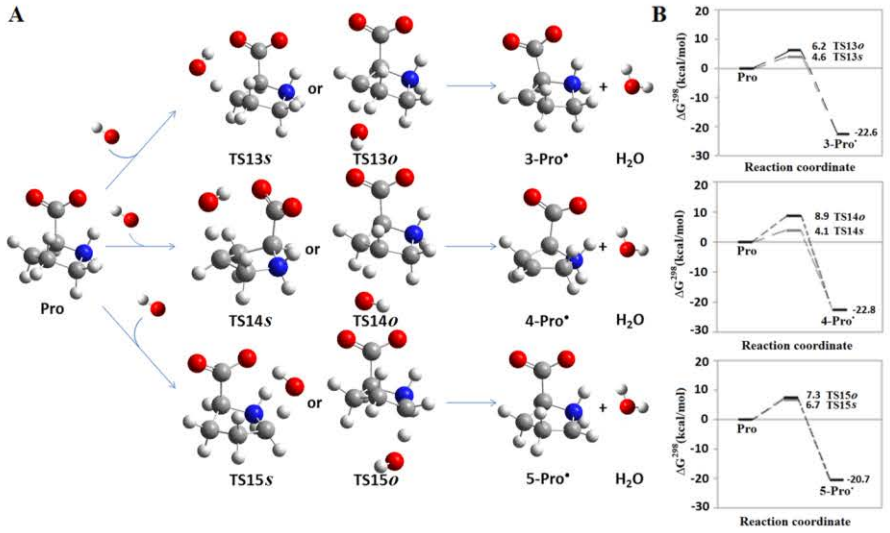


Figure 1. First H-atom abstraction from **Pro** in aqueous solution. (A) Structures for the reactants, TSs, and product radicals, considering both the attack from the carboxyl group side (s-face) and the opposite side of the ring (o-face). Intermediate complexes (IC1s) characterized are not shown here for the sake of clarity; their structures are displayed as Figure S2 in the Supporting Information. (B) Gibbs free energy profiles in aqueous solution relative to reactants for each reaction channel.

As previously anticipated based upon the BDEs values for the forming and breaking of bonds involved in the reaction coordinate for each of these channels, all of them are quite exoergic (and strongly exothermic, see Table S1 in the Supporting Information for the detailed set of data). Side-chain abstraction leading to C3/C4-centered radicals are thermodynamically preferred by ~2 kcal mol^{-1} over H extraction from C2/C5. Activation barriers are in the 4−9 kcal mol^{-1} range, corresponding to the picture of near-diffusion controlled extremely fast processes, even without considering tunneling effects[82,83] typical of this type of reaction. S-face attack is favored over the o-face one in all cases, as the result of differential stabilizing interactions (mainly HB and dipole−dipole alignments) between the approaching •OH and the $-CO_2^-$ moiety in the former plane of approach. Abstraction on C2/C5 ($\Delta G^{\ddagger}$ = 6.9 kcal mol^{-1} for hydrogen removal at C2, not shown in Figure 1) requires surmounting activation barriers 2 kcal mol^{-1} higher with respect to the attack on C3/C4. This kinetic preference toward β/γ channels agrees both with the observed side-chain selectivity and with the reduced site-specificity typical of processes involving highly reactive molecules such as •OH. Whereas thermodynamics mirrors kinetics for s-face H removal (the more exoergic the attack, the lower the barrier height) not a clear trend emerges from the o-face H-abstraction channels, pointing out the relevancy of the interactions established between the carboxylate group and •OH in defining the fate of the process. No basis is found here in support of the assumption that the relative reactivity of the H-abstraction sites in **Pro** would correlate their BDEs ordering, as previously proposed by several authors. Prereactive complexes characterized for these channels are found to be stable in terms of enthalpy relative to reactants by amounts of the order of 7 and 1−2 kcal mol^{-1} for s-attack and o-attack orientations, respectively. Once again this results from differential stabilizing interactions established between •OH and the carboxylate moiety (see the structures of both types of encounter complexes in Figure S2 of the Supporting Information). Unfavorable entropic contributions turn all these prereactive complexes unstable in terms of free energy at 298 K by 1.5−6.6 kcal mol^{-1} (Table S1 of the Supporting Information), providing evidence to discard an eventual role for these intermediate species acting as kinetic traps, a result recently found by Scheiner and Kar[49] for encounter complexes intermediating H-abstraction by •OH from a leucine dipeptide defining the selectivity and specificity of such a H-abstraction.

C-centered **Pro•** radical species obtained as the outcome of Step 1 in zwitterionic form are considerably more stable (20.7−22.8 kcal mol^{-1}, see Figure 1B) than the isolated reactants, with a clear thermodynamic preference toward production of C3/C4 radicals. This fact debilitates in principle the hypothesis previously advanced by several authors[15,61] assuming that **5-Pro•** would be the most stable product for the first H-abstraction. On the other hand, these results are more in line with the conclusions of Nukuna et al.[61] on the preferred reaction sites of **Pro** derived from ^{2}H NMR detection of ^{1}H/^{2}H exchange induced by •OH radicals. The γ(C4)-hydrogen atoms are shown to be more prone to exchange than the δ(C5)-H atoms, which in turn would exchange in a larger extent than the β(C3)-H, whereas α(C2)-H exchange is absent.[61]

Looking for clues to reconcile all these observations, the data collected in Table 1 clearly show an ordering in stability among

Table 1. Relative Stability[a] of the C-Centered Pro• Radicals (Neutral and Zwitterion Hydrogen-Bonded Forms) and Reactivity of the Zwitterionic (zw) Species[b]

	relative ΔG^{298}		hardness (η)
Pro•	neutral	zw	zw
C2	**0.0**	17.0	0.1509
C3	16.7	**16.9**	0.1322
C4	16.5	**16.7**	0.1292
C5	**13.0**	18.7	0.1388

[a]Gibbs free energies calculated at the PCM(water)/UM06/6-31G-(d,p) level and 298.15 K with respect to the most stable C-centered **Pro•** radical isomer, in kilocalories per mole. [b]Hardness was calculated as in the conceptual DFT framework from the energies of the Kohn−Sham frontier orbitals calculated at the same level, in a.u.

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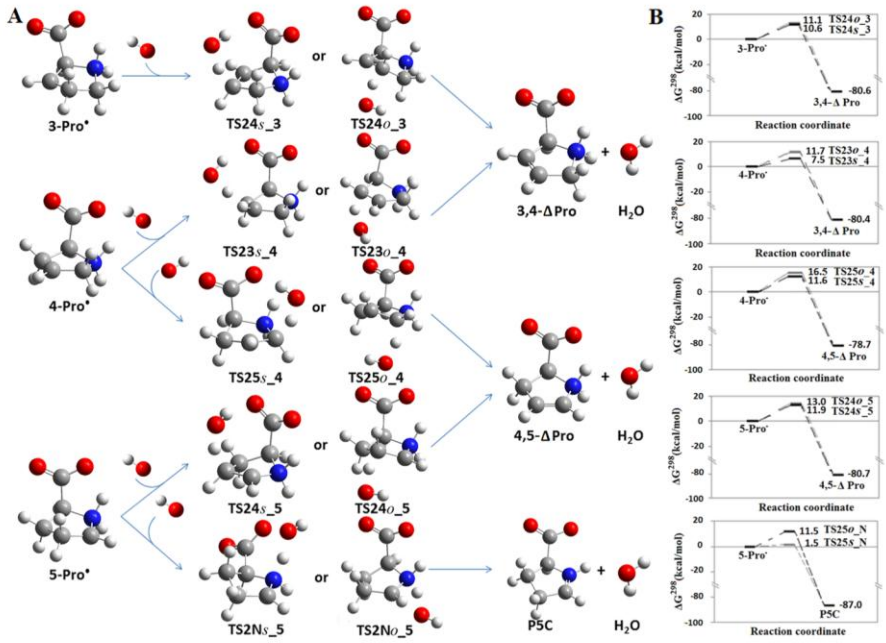

Figure 2. Second H-atom abstraction from **Pro•** C-centered radicals in aqueous solution. (A) Structures for the reactants, TSs, and unsaturated products considering both the attack from the carboxylate side (s-face) and the opposite side of the ring (o-face). Intermediate complexes (IC2s) are not shown here for the sake of clarity; their structures are displayed as Supporting Information in Figure S3. (B) Energetic profiles in aqueous solution are relative to reactants for each of the ten reaction channels considered.

such short-lived open-shell zwitterions that can be significantly altered in passing to the corresponding neutral counterparts through an internal proton transfer that retains the intramolecular HB. Recalling concepts such as the captodative effect[39] and −N(H) lone-pair delocalization in the neutral form makes it possible to explain the differential stabilization of neutral **C2-Pro•** and **C5-Pro•**, which become the most stable radicals in such a form. Variations in pH and ionic strength in environments typical of stressed plants could be influencing the preference among each pair of neutral and zwitterion radical species making difficult a direct comparison of experiments. This could be a key aspect to keep in mind in order to reconcile observations and conclusions coming from different fields.

Concerning further reactivity of these species, in a similar way Gly• and Ala• amino acid radicals produced by •OH attack have been recently shown by Owen et al.[53] to reconvert into their corresponding closed-shell species by H reabstraction from H_2O_2 (involving barriers of ca. 17 kcal mol^{-1}), **Pro•** species might be turned back into **Pro** as follows:

$$\mathbf{Pro}^{\bullet} + H_2O_2 \rightarrow \mathbf{Pro} + HO_2^{\bullet} \quad (1)$$

At physiological pH, the hydroperoxyl radical ($HO_2^{\bullet}$, the weak conjugated acid of $O_2^{\bullet-}$ with a pK_a value of 4.8) obtained as an intermediate in eq 1 is able to promote superoxide dismutation through both spontaneous and superoxide dismutase catalyzed pathways (with respective rate constants of approximately 2×10^5 and 2×10^9 M^{-1} s^{-1})[84] as follows:

$$O_2^{\bullet-} + HO_2^{\bullet} + H^+ \rightarrow H_2O_2 + O_2 \quad (2)$$

acting thus H_2O_2 as a catalyst for the consumption of **Pro•** and $O_2^{\bullet-}$ as shown in eq 3:

$$O_2^{\bullet-} + \mathbf{Pro}^{\bullet} + H^+ \rightarrow \mathbf{Pro} + O_2 \quad (3)$$

Finally, C-centered **Pro•** radicals might also be repaired by H-atom donation from −SH groups or might react with O_2 under aerobic conditions to produce peroxyl radicals that can further dehydrate to yield the expected species hydroxyproline.[42,85]

3.2. Going Further Pro• Radicals: Successive H-Abstraction Routes Leading to Relevant Species Pro Metabolism in Stressed Plants. Our exhaustive search on the triplet and singlet potential energy surfaces in aqueous solution conducted from both sides of attack by •OH over every C-centered radical obtained from Step 1 was unable to locate any TS structure for these processes globally identified as Step 2a in Scheme I. Assisted paths including one and two explicit water molecules acting as catalysts were also explored with the same outcome. In spite of that, as this kind of radical−radical recombination process is expected to display very low barriers and to be strongly exothermic, reaction free-energy values of respectively −85.9, −84.6, and −88.1 kcal mol^{-1} obtained for the favored s-face attacks by •OH over C3, C4, and C5 **Pro•** radicals suggest a first feasible channel leading to **Hyp**.

The goal of transforming the highly reactive C-centered radicals into less deleterious species can also be reached by a successive H-abstraction process from C or N atoms adjacent to the initial radical site, giving place to spontaneous formation of unsaturated cyclic species (Scheme I, Step 2b). Figure 2 collects the structural features and energetic profiles for reactants, TSs, and products (**3,4-Δ-Pro**, **4,5-Δ-Pro**, and **P5C**) for each of the ten reaction channels explored as Step 2b, including •OH attack at adjacent positions from **C3-Pro•**, **C4-Pro•**, and **C5-Pro•** from both faces of approaching the pyrrolidine ring (s and o channels). Taking into account the higher barrier obtained for a first H-abstraction at C2 and the fact that the present study focuses on the search of a possible link between the protective role of proline and the generation of **Hyp**, successive H-abstractions from C2 radical are not further discussed here.

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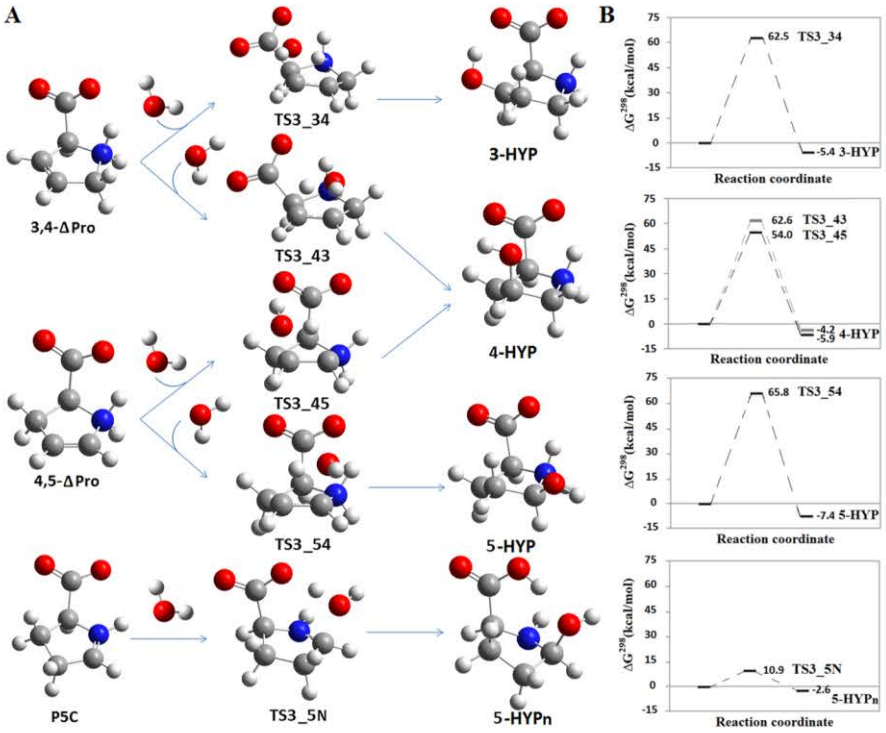

Figure 3. Structure and energetics of the species involved in hydration of **Δ-Pro**. (A) Structure for the reactants and TSs and hydroxyproline isomers (**3-Hyp**, **4-Hyp**, and **5-Hyp**/**Hyp$_n$**) corresponding for the attack from the carboxylate group side of the ring (s-face). Encounter complexes (IC3s) are not shown here for the sake of clarity; their structures are displayed as Supporting Information in Figure S5. (B) Gibbs free energy profiles in aqueous solution at 298.15 K are relative to reactants for each of the five channels explored.

Concerning the prereactive complexes (IC2), those corresponding to the second H-abstraction by $^{\bullet}$OH approaching from the s-face are fairly more stable than those corresponding to the o-face attack (see details in Figure S3 and Table S1 in the Supporting Information). This is mainly due to $^{\bullet}$OH··· carboxylate hydrogen-bonding present in all the s-face encounter complexes but in IC2Ns_5. In spite of this observation, and as for IC1 complexes in Step 1, the stabilization gained in forming IC2s is completely overcome by the unfavorable entropic cost of arranging reactants in more organized structures. For this reason all the barrier heights reported in Figure 2B and discussed here correspond to the bimolecular processes, calculated with respect to the reactant separated at the infinite.

The activation barriers obtained for the second H-abstraction are now in the 1.5−16.9 kcal mol^{-1} range, showing a more pronounced variability among different channels. Once again, s-face processes are all favored with respect to the corresponding o-face reaction paths. This remarks the relevance of establishing intermolecular HBs for achieving a differential stabilization of the TSs that leads to reduced heights at the corresponding barriers. Starting from the major products from Step 1 (**4-Pro$^{\bullet}$** and **3-Pro$^{\bullet}$**) the kinetically preferred path leads to formation of **3,4-Δ-Pro** with an associated barrier of 7.5 kcal mol^{-1} at 298.15 K in aqueous solution. This value competes well against the mechanistic alternative for converting **Pro$^{\bullet}$** back to **Pro** under H_2O_2 catalysis (with values of $\Delta G^{\ddagger}$ = 10.7 kcal mol^{-1} and ΔG_{rxn} = −10.9 kcal mol^{-1} for the process of reconverting **4-Pro$^{\bullet}$**, as the main product from Step 1; see the structure of the species involved in this channel in Figure S4 in the Supporting Information) emerging here as a more favorable channel that enables the envisioning of another feasible route to reach the eventual production of **Hyp** isomers.

Even if obtained as a secondary product from Step 1, a very interesting mechanistic alternative emerges from the **5-Pro$^{\bullet}$** zwitterion: a successive H abstraction at the N-protonated site produces Δ^1-pyrrolidin-5-carboxylate (**P5C**) through a near-barrierless $^{\bullet}$OH attack ($\Delta G^{\ddagger}$ = 1.5 kcal mol^{-1}). **P5C** is a well-known precursor in the biosynthesis of proline,[9,86,87] also recently identified by Kenttämaa and co-workers as a product of two consecutive H-abstractions initiated by charged phenyl radicals on this amino acid.[70] Moreover, conversion of **P5C** into **Pro** is known to be efficiently catalyzed by pyrroline 5-carboxylate reductase (**P5CR**, EC 1.5.1.2),[88] an enzyme induced under stress conditions in plants[9,89,90] that consumes one **NADPH** molecule per **P5C** (see Scheme II in Section 3.4).

3.3. Finding the Missing Link toward Hyp: Is It Produced by H_2O/$^{\bullet}$OH Addition on Δ-Pro? Aiming to find evidence for a plausible mechanistic proposal linking the protective role of proline and the hypothesized production of **5-Hyp** under plant stress, we explored several channels for hydration of **Δ-Pro** species by direct addition of water on the C═C and C═N unsaturation (identified as Step 3a in Scheme I). On the basis of the marked preference toward s-face attacks shown by **Pro** and **Pro$^{\bullet}$** in Steps 1 and 2b, this face was the only one systematically explored in this case. Figure 3 displays the structural features and energetic profiles for reactants, TSs, and products (**3-Hyp**, **4-Hyp**, and **5-Hyp** zwitterions and neutral species; see explanation below) for each of the five reaction channels explored as Step 3a, resulting from considering both water asymmetric additions on C3═C4 and C4═C5 and a single addition on C5═N.

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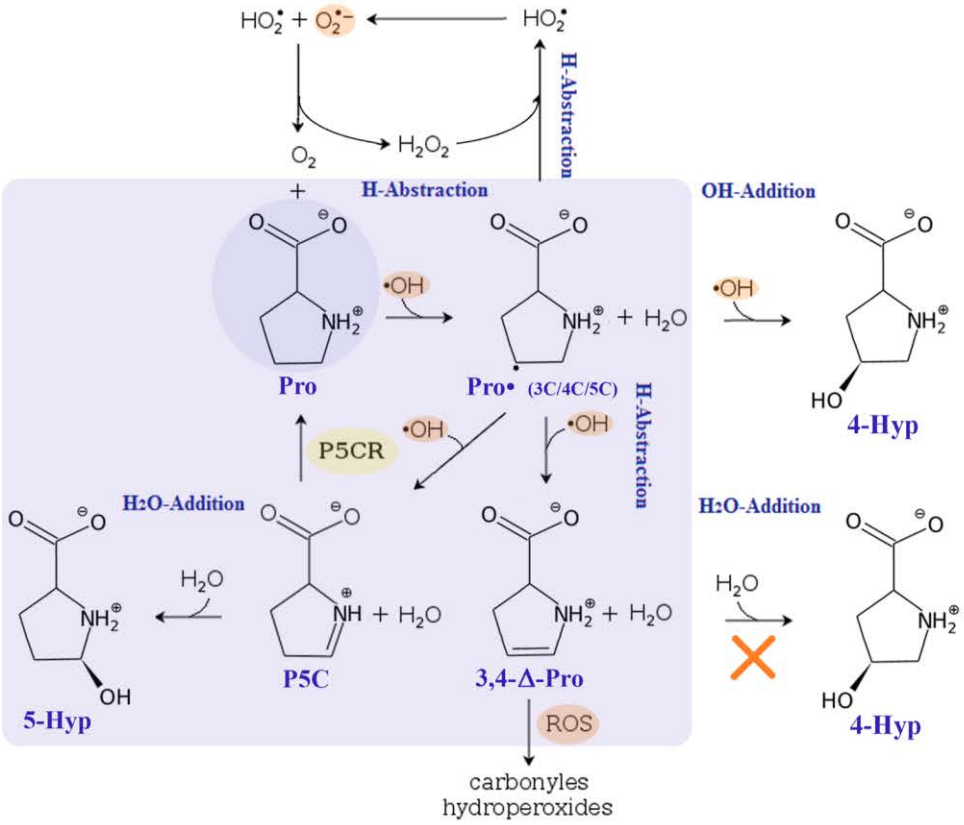
Scheme II. Feasibility of the Pathways Explored for Assessing Proline Activity As a •OH Scavenger. The Main Reactions Consuming •OH by Both Proline (Pro) and Its Derivatives (Pro•, Δ-Pro/P5C, and Hyp) are Identified by Horizontal and Vertical Blue Labels



Hydration by direct addition of a water molecule on the C=C bonds is associated to very high barrier heights (ca. 54−66 kcal mol^{-1}), making it possible to discard these reactions as feasible in the absence of a catalyst. On the other hand, direct hydration on the C=N bond in **P5C** appears as a strongly favored reaction channel with a low activation barrier of 10.9 kcal mol^{-1}. This provides the mechanistic missing link we sought for the connection of •OH scavenging by **Pro** with formation of 5-hydroxyproline (**5-Hyp$_n$** in Figure 3A). Notice that in contrast with the rest of the processes studied as Step 3a (for which the zwitterionic form of **Hyp** is the only outcome for direct hydration on both **Δ-Pro** species) 5-hydroxyproline happens to be obtained in the neutral form from this path. A closer inspection on the nature of the reactants, transition states characterized for each hydration channel, and their corresponding IRC reaction paths can be useful to shed light on the differences in the mechanism of each reaction described at a very detailed level.

In the first place, water addition over C=N at **P5C** zwitterion can be depicted as a process assisted by the proximal COO^- moiety acting as a Brönsted base which captures a proton from the approaching H_2O molecule while the emerging hydroxyl anion attacks C5 in a concerted way. This enables the protonated imino moiety to develop a lone pair on N as the reaction proceeds breaking the C=N double bond, while the initial intermolecular hydrogen bond HOH···$^-$O(C=O) evolves to an intramolecular C5-(H)O···HO(C=O) HB as in 5-**Hyp$_n$** (see Figure 3A, bottom) once the coupled proton transfer/HO^- addition are completed. Second, concerning the reaction of water on C=C at the different **Δ-Pro** zwitterions, all four channels here characterized (see Figure 3A) correspond to a concerted process, with a water molecule simultaneously attacking the two adjacent C(sp^2) atoms in order to form two new bonds [C(sp^2)···O(H) and C···H] while one H−O bond in H_2O is broken. Reactants and **Hyp** zwitterionic products are thus interconnected, passing through cyclic 4-centered transition state structures, which are quite tensioned and extremely unstable, a fact reflected in the high values obtained for the corresponding barrier heights (see Figure 3B). Taking into account that assistant water molecules acting as bifunctional catalysts have been shown to accelerate hydration and related condensation processes on unsaturated moieties,[91−93] alternative channels including one ancillary water in the reaction coordinate were also explored. Thus, four more relaxed 6-centered cyclic TSs were located, still resulting in associated to noncompetitive barrier heights (50 kcal mol^{-1} or higher, see the last four rows in Table S1 and Figure S6 in the Supporting Information) with respect to hydration of **P5C** linking to **5-Hyp**.

In closing the mechanistic exploration here undertaken, addition of •OH on C=C **Δ-Pro** derivatives (Scheme I, Step 3b) was also examined to assess whether **Hyp** production might be feasible through this alternative pathway as a way of further scavenging •OH. Only a couple of first-order saddle points could be found at the DFT level on the doublet potential energy surface corresponding to the approach of •OH to C3/C5 from the s-face to yield **3-Hyp•**/**5-Hyp•** radicals, and both of them were affected by significant spin contamination (expected values for $S^2 \geq 0.77-0.78$). Considering these structures and their associated energetics untrustworthy, a more rigorous characterization of the

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Table 2. Selection of PCM-UM06/6-31G(d,p) Energetics at 298.15 K in Aqueous Solution[a] for the Principal and Secondary Chemical Pathways in the Overall Mechanism of Proline as •OH Scavenger

step and type of reaction	species in the main path	$\Delta G^{\ddagger b}$	$\Delta G_{rxn}{}^{c}$	species in the secondary path	$\Delta G^{\ddagger b}$	$\Delta G_{rxn}{}^{c}$
1: H abstraction	**3-Pro•/4-Pro•**	4.6/4.1	−22.6/−22.8	**5-Pro•**	6.7	−20.7
2a: **Pro•**/•OH assoc.	**3-Hyp$_{zw}$/4-Hyp$_{zw}$**	−[d]	−85.9/−84.6	**5-Hyp$_{zw}$**	−[d]	−88.1
2b: H abstraction	**3,4-Δ-Pro**	7.5	−80.4	**P5C**	1.5	−87.0
3a: hydration	–	–	–	**5-Hyp$_{n}$**	10.9	−2.6

[a]Relative Gibbs free energies calculated at the DFT/PCM level taken with respect to reactants for each step separated to the infinite. [b]Activation barrier height. [c]Reaction free energy. [d]Barrierless radical−radical association processes.

species involved in these two channels was conducted at the PCM(water)/UCCSD/cc-pVDZ level. No first-order saddle points were localized at this level for •OH addition on both C3 and C5. At the same time, it is worth recalling that these **Δ-Pro** derivatives are likely to exert their antioxidant role through interactions of their π-electron density with other ROS such as $^1O_2$ or $O_2^{\bullet-}$, forming carbonyls and hydroperoxides.[40−43,85]

3.4. Overall Mechanism for •OH Scavenging by Proline, Biological Significance in Plant Stress, and a Pro-Pro Cycle Proposal. Key aspects emerging from the extended mechanistic analysis here conducted are summarized in Scheme II. The scheme discriminates among feasible and not feasible reaction channels in a physicochemical and biological context and depicts main structural features of the species interconnected in the •OH scavenging network. A selection of data on energetics for the most relevant channels and intermediates is also collected in Table 2.

The mechanistic evidence collected here based upon DFT/PCM modeling of a network of reaction pathways provides strong support to the concept advanced earlier by Smirnoff and Cumbes[14] on proline having a protective role in stressed plants by acting as a •OH scavenger. This would be accomplished through a complex mechanism which in overview involves consumption of a minimum of two hydroxyl radicals per amino acid molecule and enables the recovery of the original **Pro** by enzymatic recycling of chemically produced **P5C**.

Such an activity would take place in the cellular environment through three main kinds of chemical transformations, including (a) initiation by H-abstraction from **Pro** by •OH with the evidence supporting kinetic and thermodynamic preference toward production of **4-Pro•/3-Pro•** that can be promptly terminated by **Pro•**/•OH radical-pair association, mainly leading to **3-Hyp$_{zw}$/4-Hyp$_{zw}$/5-Hyp$_{zw}$**; (b) a successive H-abstraction step from the intermediate **Pro•** radicals, competitive with termination, with the evidence supporting kinetic and thermodynamic preference toward production of **3,4-Δ-Pro** as a main path, accompanied by a secondary pathway relevant to chemical production of **P5C** passing through **5-Pro•**; and (c) direct hydration of **P5C** to produce **5-Hyp$_{n}$** in competition with enzymatic recycling of **P5C** back to **Pro** as catalyzed by **P5CR/NADPH.**

Through this series of transformations, a molecule of **Pro** would be intrinsically successful in progressively converting deleterious •OH radicals into less-harmful species (**Pro**, **Δ-Pro**, **Hyp$_{zw/n}$**, and water), exerting a protective role in general terms.

To consider all these findings in a proper biological perspective, completing the scenario for an antioxidant activity of proline in stressed plants would require keeping in mind that whereas •OH is one of the most reactive, and thus one of the least selective, radical species with a relatively narrow range of rate constants (k = 10^7−10^{10} M^{-1} s^{-1}) for reacting with amino acids,[85,94] under normal conditions **Pro** is one of the least abundant of them. In a first glance, this might induce one to think that **Pro** would not be the best candidate for exerting an antioxidant activity against •OH. Two more key elements have to be taken into account in composing the picture for sustaining such an activity. The first one is the aforementioned increase in **Pro** concentration experienced by plants under any kind of stress (raising up to 80% of the total pool of amino acids[7−9,15]); the second one is colocalization of both species in the cytosol and chloroplasts, where •OH is commonly produced as a consequence of disruption in the electron transport of photosystems. At these locations **Pro** accumulation reaches, even under nonstressful conditions, concentrations of at least 160 mM,[8,95] becoming considerably more abundant than every other common antioxidant (i.e., 1−5 mM for glutathione and 5−20 mM for ascorbato).[1,2,9] Moreover, cytosol and chloroplasts also are subcellular compartments where **Pro** biosynthesis enzymes such as **P5CR** are localized.[9,96] All these elements provide solid significance to our mechanistic findings in the precise context of proline's accumulation in stressed plants.

Finally, we propose here for the first time a **Pro**-(**P5C**)-**Pro** cycle as depicted in Figure 4, where the efficiency of the

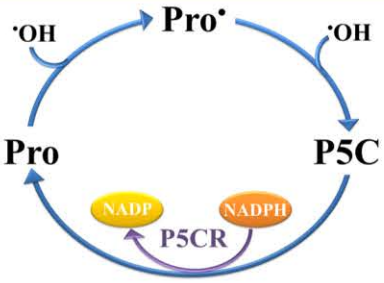

Figure 4. Pro-Pro cycle. Proline captures a first •OH by H-abstraction on C5, followed by a second H-abstraction which also captures •OH, yielding **P5C**. **P5C** is then recycled back to **Pro** by the action of the **P5CR/NADPH** enzymatic system.

enzymatic action exerted by **P5CR** in converting toxic **P5C** back into **Pro**[97] is coupled to the tandem of very fast chemical reactions (in the near-diffusion limit) capturing •OH by **Pro**/**5C-Pro•** to yield **P5C** and two water molecules. As for other enzymes in the biosynthetic pathway of proline, under osmotic stress in which **Pro** accumulation takes place, **P5CR** is also overexpressed[9,90] and **NADPH** is accumulated as a result of inhibition of the Calvin cycle.[98] The enhanced activity of **P5CR** under plant stress will thus extremely favor a prompt recovery of **Pro** from **P5C** originated through chemical scavenging of •OH, acting as a driving force increasing the relevance of this cycle. Although production of **5-Hyp$_{n}$** by **P5C** hydration is characterized here as a very fast chemical process, because toxic **P5C** would be present in very low concentrations by the effect of this cycle, this channel

would yield a very low amount of 5-hydroxyproline, a fact that might explain the lack of experimental evidence of its formation.

4. CONCLUSIONS

Mechanistic evidence was collected here by using DFT/PCM models to sustain a role for proline as a protective $^{\bullet}$OH scavenger under stress in plants. At least two $^{\bullet}$OH radicals are consumed per **Pro** molecule, leading to less deleterious species through strongly exothermic and exergonic channels with very low reaction barriers (mostly in the near-diffusion limit). A clear preference for attacking **Pro** from the carboxylate face (s-face) is noticed, explained by inter- and intramolecular hydrogen-bond interactions that differentially stabilize the corresponding transition states. Our global mechanistic proposal includes two transformation pathways, a main one connecting **Pro** to **3,4-Δ-Pro** through two consecutive H-abstraction processes, the second one in competition with a chance of early termination through barrierless and strongly exothermic radical−radical associative processes leading to $\mathbf{Hyp}_{zw}$. A secondary path connects to **P5C** as an intermediate capable of evolving to 5-$\mathbf{Hyp}_n$ by hydration, although the action of the **P5CR/NADPH** system (overexpressed under plant stress and colocalized at the same compartment where $^{\bullet}$OH is produced and **Pro** is accumulated) reconverts **P5C** to **Pro**. Such an enzymatic process elicits **P5C** toxicity in the cell, contributing to the reinforcement of **Pro** accumulation and turning hydration leading to **5-Hyp** a quite unlikely channel in photosynthetic tissues. All this evidence gives a solid base for hypothesizing a **Pro**-(**P5C**)-**Pro** cycle, emerging from this study as an original proposal of impact in the field of research in plant stress, whereas consumption of **NADPH** by this cycle would contribute to support a role for **Pro** in maintaining redox homeostasis.

■ ASSOCIATED CONTENT

Supporting Information

Full Gaussian09 reference; differences between neutral and zwitterionic **Pro**; structure of the intermediate complexes for Steps 1, 2b, and 3a; complete table of relative energetics (enthalpy and free energy); structure and energetics for the species involved in reconverting **4C-Pro**$^{\bullet}$ to **4C-Pro** by H-abstraction from H_2O_2 and in the water-assisted hydration of **Δ-Pro** species (Step $3a_w$). This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

S.S. and E.L.C. designed the strategy of modeling, performed calculations, and interpreted the physicochemical results. S.S., O.B., and J.M. contributed the biological background of the problem. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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Supporting Information

Molecular Mechanisms for the Reaction Between ·OH Radicals and Proline: Insights on the Role as ROS Scavenger in Plant Stress

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Complete reference for Gaussian09 [77]

M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, B. Mennucci, G. A. Petersson, H. Nakatsuji, M. Caricato, X. Li, H. P. Hratchian, A. F. Izmaylov, J. Bloino, G. Zheng, J. L. Sonnenberg, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, J. A. Montgomery, Jr., J. E. Peralta, F. Ogliaro, M. Bearpark, J. J. Heyd, E. Brothers, K. N. Kudin, V. N. Staroverov, R. Kobayashi, J. Normand, K. Raghavachari, A. Rendell, J. C. Burant, S. S. Iyengar, J. Tomasi, M. Cossi, N. Rega, J. M. Millam, M. Klene, J. E. Knox, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. W. Ochterski, R. L. Martin, K. Morokuma, V. G. Zakrzewski, G. A. Voth, P. Salvador, J. J. Dannenberg, S. Dapprich, A. D. Daniels, O. Farkas, J. B. Foresman, J. V. Ortiz, J. Cioslowski, and D. J. Fox, *Gaussian 09, Revision A.02*, Gaussian, Inc., Wallingford CT, **2009**.

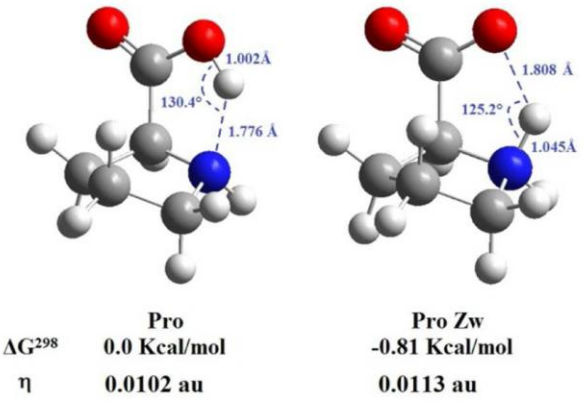

Figure S1 – Structure, relative stability and reactivity (expressed as hardness, η) corresponding to the stable neutral and *zwitterion* free L-proline isomers (**Pro** and **Pro$_{zw}$**, respectively) displaying an intramolecular hydrogen-bond, as predicted at the IEF-PCM(water)/M06-2X/6-31G(d,p) level.

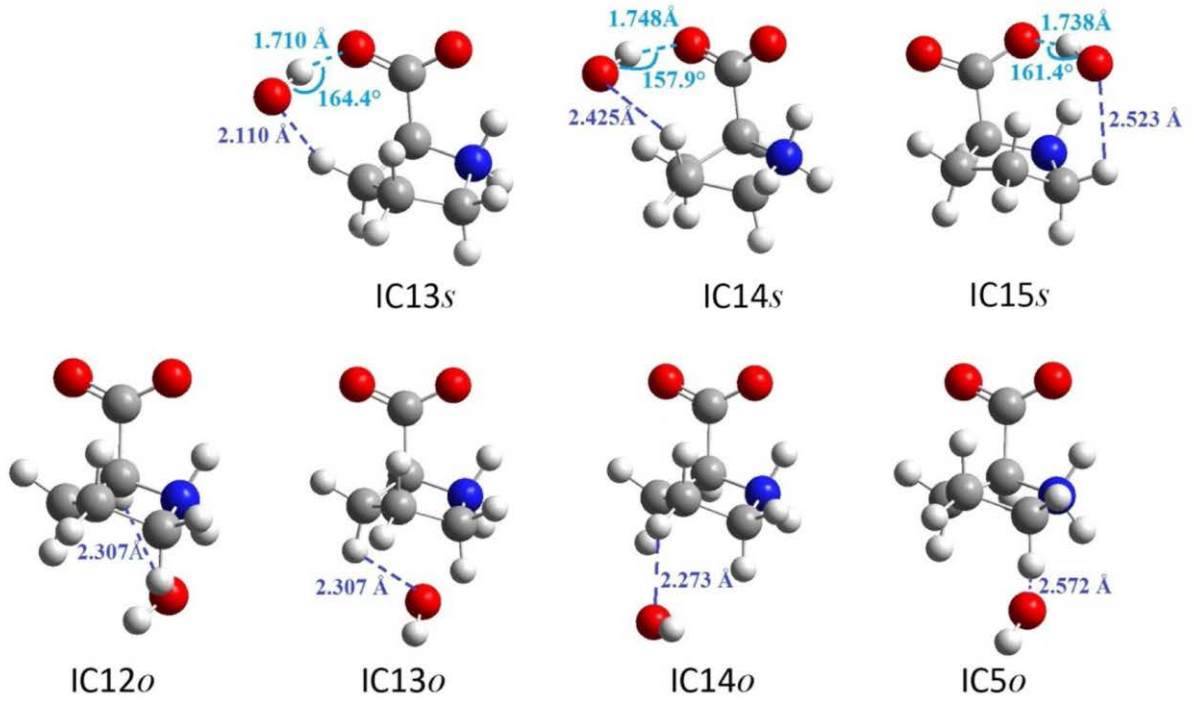

Figure S2 – Structure of the pre-reactive intermediate complexes (IC1s) characterized for each reaction channel corresponding to the first H-abstraction process, identified as **Step 1.**

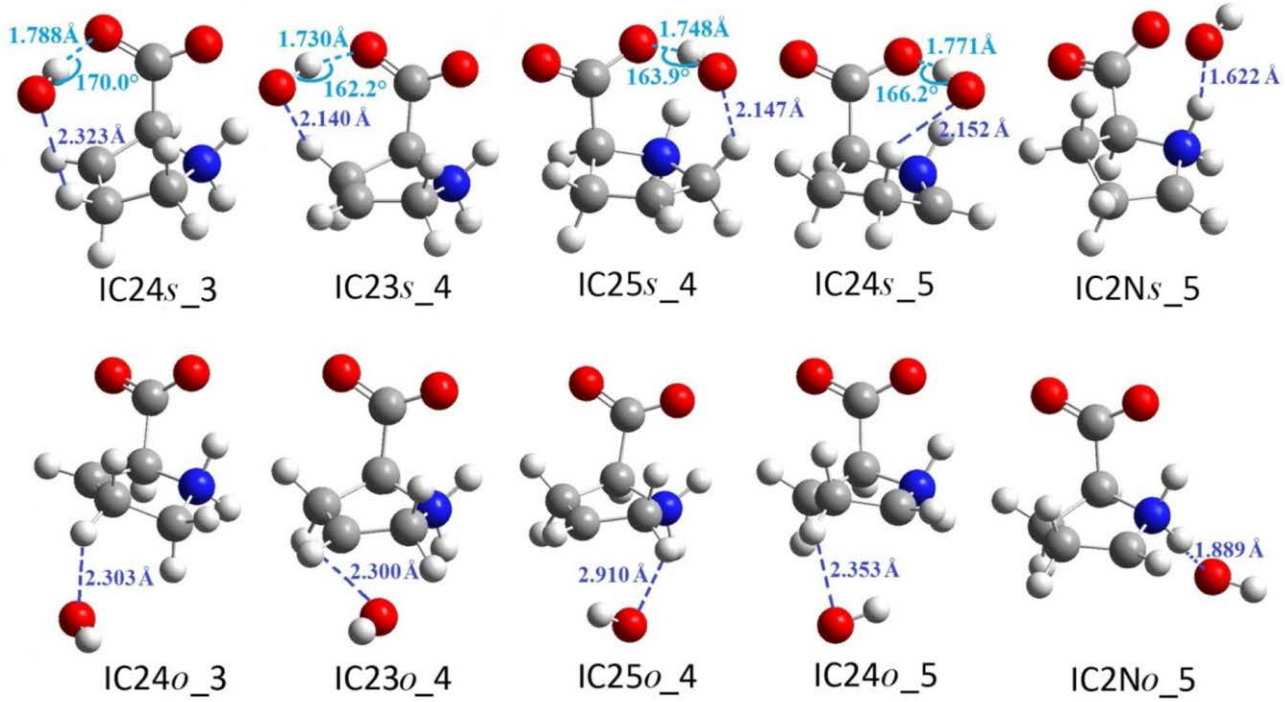

Figure S3 – Structure of the pre-reactive intermediate complexes (IC2s) characterized for each reaction channel corresponding to the successive H-abstraction process (**Step 2b**) at the different **Pro˙** radicals.

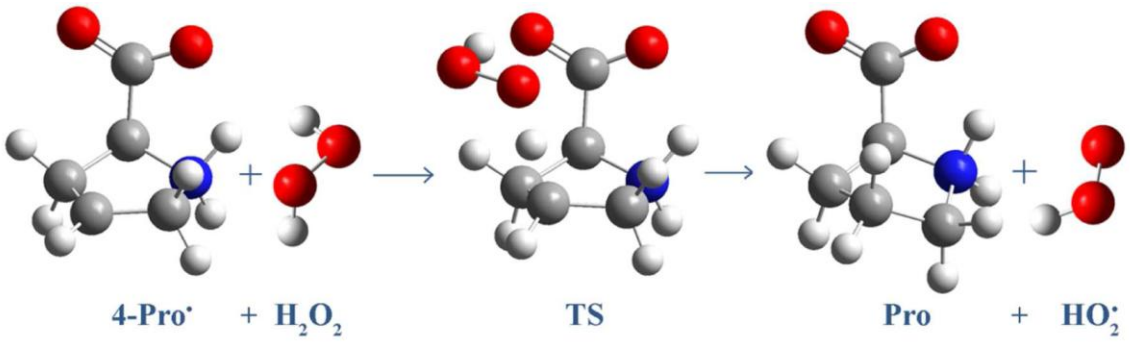

Figure S4 – Reconversion of 4-Pro$^{\bullet}$ to Pro by H-abstraction from H_2O_2. Structures for reactants, TSs and products (**Pro** in the *zwitterionic form*) as obtained at the DFT/PCM level employed in this work for the reaction proceeding from the carboxylate face of the ring (*s-face*). The reaction is quite exergonic (ΔG_{rxn} = -10.9 kcal.mol^{-1} at 298 K) passing through a quasi-linear transition state implying a free energy barrier in solution of $\Delta G^{\#}$ = 10.7 kcal.mol^{-1} at 298 K, a value that makes this channel uncompetitive with the second *H-abstraction* by **$^{\bullet}$OH** leading to unsaturated **Δ-Pro** species and/or radical-radical associative processes yielding **Hyp**.

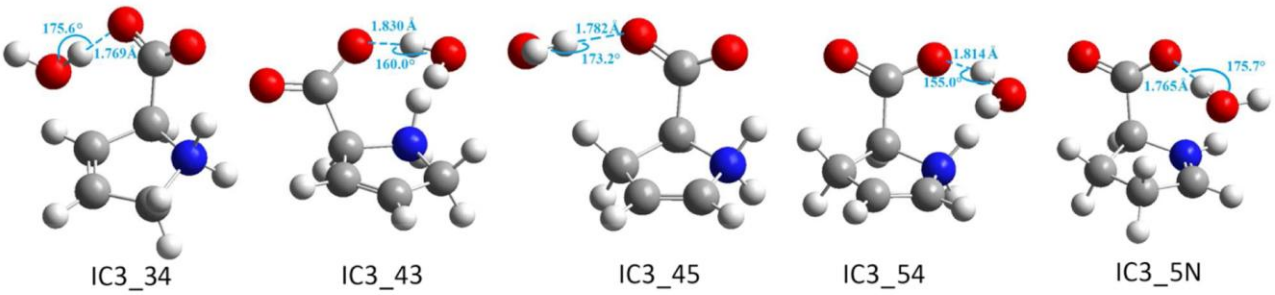

Figure S5 – Structure of the pre-reactive intermediate complexes (IC3s) characterized for each reaction channel corresponding to direct hydration on the unsaturated **Δ-Pro** intermediates, **Step 3a.**

Table S1 – Relative energetics[a] for the species involved in Steps 1-3 in terms of enthalpies and free energies (in bold type) in aqueous solution at 298.15 K in kcal/mol

Step	Site/face of attack	Pre-reactive complex	Unimolecular barrier	Bimolecular barrier	Reaction energy
1	2	-2.6/**5.7**	1.1/**1.2**	-1.5/**6.9**	-20.1/**-22.3**
	3s	-6.7/**2.6**	1.7/**2.0**	-5.1/**4.6**	-20.1/**-22.6**
	3o	-2.1/**6.5**	-0.9/**-0.3**	-3.0/**6.2**	
	4s	-6.7/**2.7**	1.6/**1.5**	-5.1/**4.1**	-20.1/**-22.8**
	4o	-1.0/**6.6**	1.0/**2.3**	0.0/**8.9**	
	5s	-7.2/**1.5**	3.9/**5.2**	-3.3/**6.7**	-18.5/**-20.7**
	5o	-2.2/**6.4**	1.3/**0.9**	-0.9/**7.3**	
2a	3s	-	-	-	-98.2/**-85.9**
	4s	-	-	-	-96.8/**-84.6**
	5s	-	-	-	-100.9/**-88.1**
2b	4s→3	-7.8/**3.1**	7.7/**4.4**	-0.1/**7.5**	-80.0/**-80.4**
	4o→3	-1.3/**5.5**	3.3/**6.2**	2.0/**11.7**	
	3s→4	-7.1/**1.1**	4.4/**9.5**	-2.8/**10.6**	-80.8/**-80.6**
	3o→4	-4.0/**7.2**	5.8/**3.9**	1.8/**11.1**	
	5s→4	-6.8/**1.3**	7.5/**10.6**	0.7/**11.9**	-79.5/**-80.7**
	5o→4	-3.4/**8.1**	9.8/**4.9**	6.4/**13.0**	
	4s→5	-6.5/**3.6**	7.8/**8.0**	1.3/**11.6**	-81.2/**-78.7**
	4o→5	-0.9/**6.1**	4.1/**10.4**	3.2/**16.5**	
	Ns→5	-6.8/**2.5**	-2.1/**-1.0**	-8.9/**1.5**	-87.1/**-87.0**
	No→5	-2.2/**7.1**	3.9/**4.4**	1.8/**11.5**	
3a[b] ***(s-face)***	**3**-4	-5.7/**4.4**	55.9/**58.6**	50.1/**62.5**	-18.2/**-5.4**
	4-3	-4.9/**5.2**	55.4/**57.5**	50.6/**62.6**	-16.5/**-4.2**
	4-5	-4.4/**3.3**	45.5/**20.7**	41.1/**54.0**	-17.9/**-5.9**
	5-4	-3.7/**6.8**	31.1/**58.9**	27.4/**65.8**	-19.7/**-7.4**
	5-N	-5.5/**4.5**	4.0/**6.4**	-1.5/**10.9**	-15.4/**-2.6**
3aw[c] ***(s-face)***	3w-4	-2.5/**17.4**	37.7/**40.8**	35.2/**58.2**	-18.2/**-5.4**
	4w-3	-4.4 /**15.6**	38.8/**42.7**	34.3/**58.3**	-16.5/**-4.2**
	4w-5	-8.7 /**11.5**	37.5/**41.0**	28.8/**52.5**	-17.9/**-5.9**
	5w-4	-5.1 /**15.5**	40.0/**42.6**	34.9/**58.1**	-19.7/**-7.4**

[a]Calculated respect to reactants of each channel separated at the infinite. [b]3a: direct hydration. [c]3aw: water assisted hydration.

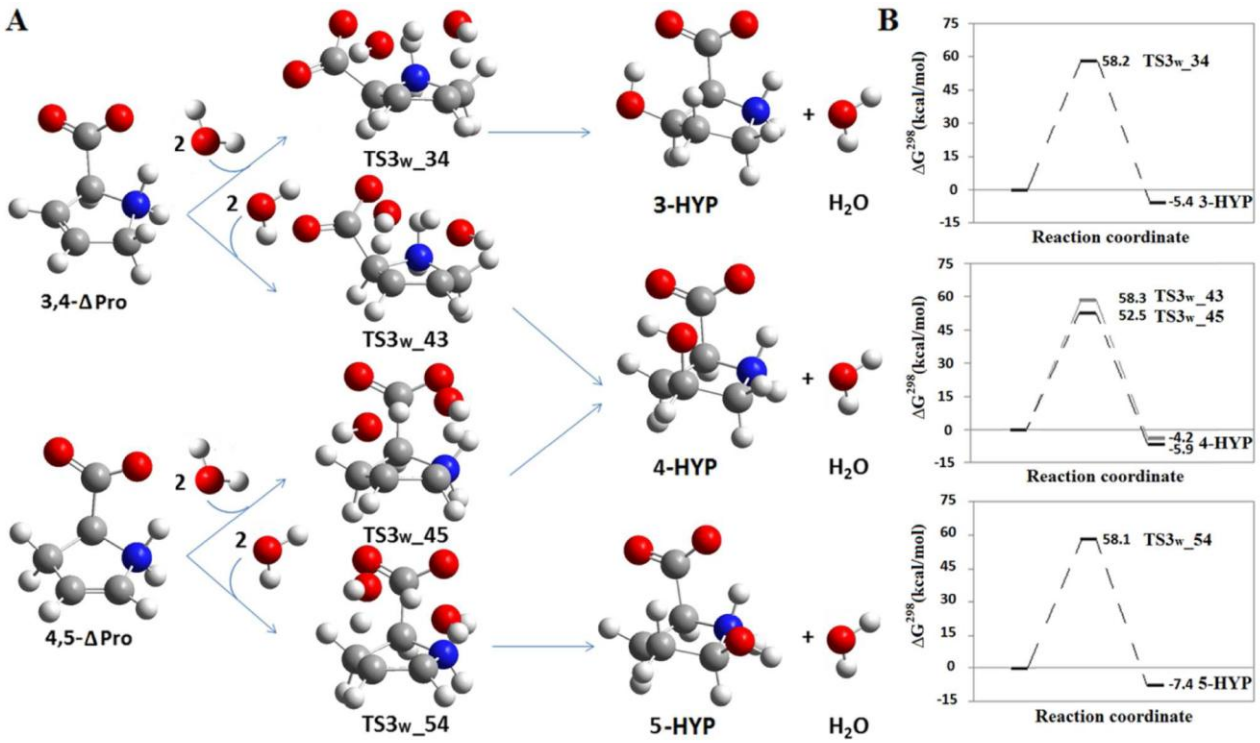

Figure S6 – Water assisted addition of H_2O to C=C in Δ-Pro species to form Hyp. (A) Structure for reactants, TSs and hydroxyproline regioisomers (**3-Hyp**, **4-Hyp**, and **5-Hyp**) in the *zwitterionic form* for the reaction proceeding from the carboxylate face of the ring (*s-face*). **(B)** Gibbs' free energy profiles in aqueous solution at 298.15 K relative to reactants for each of the three water-assisted channels explored.

III. Formación de GABA como consecuencia de las reacciones Pro - ·OH

Al caracterizar las reacciones entre el ·OH y la prolina (Cápitulo 2, sección II) sólo se consideraron las abstracciones de H sobre los C, porque el objetivo era establecer la factibilidad de formación de hidroxiprolina. En el artículo que se presenta en esta sección se consideró la posibilidad de que el ·OH realice la primera abstracción de H en el grupo NH_2^+ (Signorelli et al., n.d.). Nuestros resultados demostraron que esta reacción tiene una barrera energética más baja que las abstracciones de H unidos a los C (3,0 kcal/mol vs 4,1-8,9 kcal/mol). De todas formas, cuando la energía de las barreras que compiten son menores a 4,5 kcal/mol no hay especificidad entre una u otra reacción, ya que todas se encuentran controladas por difusión. Lo que determinará que el ·OH reaccione con el H de la prolina unido al N (3,0 kcal/mol) o con el H unido al Cγ (4,1 kcal/mol) se debe más a la orientación con la que se encuentren estas moléculas. A diferencia de lo que sucede cuando las abstracciones ocurren sobre los H unidos al C, el electrón desapareado no queda sobre el átomo que sufrió la abstracción, sino que se moviliza espontáneamente hacia el Cα produciendo la liberación del grupo carboxilo como CO_2. La molécula producida es un pirrolín radical que mediante otra abstracción de H por ·OH, u otra molécula, puede convertirse en el sustrato de la enzima Δ^1-pirrolín deshidrogenasa que lo convierte en ácido γ-aminobutírico (GABA). La acumulación de esta molécula en plantas ocurre en diferentes condiciones de estrés, y al igual que a la prolina se han planteado diversas hipótesis sobre su rol durante el estrés.

La vía principal de síntesis de GABA es a partir del glutamato por actividad de la glutamato deshidrogenasa. Nosotros proponemos una vía alternativa que incluye reacciones enzimáticas y no enzimáticas que colaboran con su acumulación, estableciendo una conexión entre GABA y prolina, dos moléculas que se acumulan durante el estrés en plantas.

Por otra parte, la transformación de Δ^1-pirrolín en GABA así como las reacciones derivadas de degradar GABA en el ciclo de Krebs, producen poder reductor en forma de $NADH.H^+$ y $FADH_2$. El consumo de NADPH requerido para la

síntesis-acumulación de prolina y la generación de NADH y $FADH_2$ a partir de la formación y catabolismo del GABA contribuyen con una mejor distribución del poder reductor en la planta.

Por último, considerando que las reacciones $^{\cdot}OH$ - prolina ocurren principalmente a nivel cloroplástico, el CO_2 liberado por la descarboxilación de la prolina, cuando el ataque ocurre sobre el grupo NH_2^+, podría ser utilizado en la fijación de CO_2 aportando, aunque mínimamente, al mantenimiento de la actividad fotosintética.

A su vez la fijación de CO_2 consume poder reductor en forma de NADPH y de este modo se generan $NADP^+$ para ser utilizado como aceptor final en el fotosistema evitando la generación de ROS por falta de aceptores de electrones fotosintéticos.

Nuevamente estas evidencias aportan elementos para fundamentar la hipótesis de un rol multifuncional de la prolina, con participación a nivel de la actividad fotosintética, la homeostasis redox y en protección frente al daño oxidativo.

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RESEARCH ARTICLE

Connecting Proline and γ-Aminobutyric Acid in Stressed Plants through Non-Enzymatic Reactions

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Abstract

The accumulation of proline (Pro) in plants exposed to biotic/abiotic stress is a well-documented and conserved response in most vegetal species. Stress conditions induce the overproduction of reactive oxygen species which can lead to cellular damage. In vitro assays have shown that enzyme inactivation by hydroxyl radicals (˙OH) can be avoided in presence of Pro, suggesting that this amino acid could act as an ˙OH scavenger. We applied Density Functional Theory coupled with a polarizable continuum model to elucidate how Pro reacts with ·OH. In this work we suggest that Pro reacts favourably with ˙OH by H–abstraction on the amine group. This reaction produces the spontaneous decarboxylation of Pro leading to the formation of pyrrolidin-1-yl. In turn, pyrrolidin-1-yl can easily be converted to Δ^1-pyrroline, the substrate of the enzyme Δ^1-pyrroline dehydrogenase, which produces γ-aminobutyric acid (GABA). GABA and Pro are frequently accumulated in stressed plants and several protective roles have been assigned to these molecules. Thereby we present an alternative non-enzymatic way to synthetize GABA under oxidative stress. Finally this work sheds light on a new beneficial role of Pro accumulation in the maintenance of photosynthetic activity.

Introduction

When plants are exposed to biotic/abiotic stress, damage on cellular components (proteins, lipids, carbohydrates, and DNA) increase as a result of the overproduction of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\cdot -}$), and hydroxyl radicals (˙OH) [1]. The latter is the most reactive species among ROS and can be generated *in vivo* either by Fenton's reaction, in the Haber-Weiss cycle, or through homolysis of H_2O_2 under UV radiation to which plants are highly exposed [2,3]. Despite its short lifetime, the production of ·OH has been detected in intact plants using EPR techniques [4,5].

Competing Interests: The authors have declared that no competing interests exist.

Hydrogen abstraction, addition, and electron transfer processes are the most common reaction channels for ˙OH, leading to new radicals or closed shell molecular species with lower reactivity [6].

Cellular defense against ROS can benefit from either enzymatic or non-enzymatic antioxidant processes. Proline (Pro) has been considered to be involved in the non-enzymatic antioxidant plant defense [7,8]. Accumulation of Pro in stressed plants, up to 100 times the normal level, has been a well-known fact for more than 40 years [9]. In this condition, Pro can reach a cytosol concentrations of 120 to 230 mM (see reference [10] and references therein). Under drought and high salinity conditions, UV/Vis irradiation, oxidative stress, the presence of heavy metals, or as a response to different kind of biotic stresses [11], Pro accumulation by *de novo* synthesis has been reported to be a feature shared by a wide variety of organisms including bacteria, fungi and plants [12].

Early in 1989, Sminorff and Cumbes showed that enzyme inactivation by ˙OH can be avoided *in vitro* by the presence of Pro, proposing that this molecule might act as a ˙OH scavenger [13]. Later, it has also been suggested that Pro could protect plants from $^1O_2$ oxidation [14,15]. Indirect evidence of such protective roles, emerged comparing oxidative damage on genetically-engineered plants under stress, where transgenes were used to control the production of Pro. Under saline conditions, transgenic plants with increased production and accumulation of Pro were less affected by oxidative damage [16], while plants genetically-unable to produce Pro exhibited a significantly lower tolerance to stress [17]. Recently, direct evidences showed that Pro does not quench singlet oxygen ($^1O_2$), as it was thought during several years, concluding that the protective role of Pro against oxidative damage, observed in several plants, could be related to the ˙OH scavenger activity [18].

A pioneer work of Amici *et al.* suggested that ˙OH reacts with Pro forming 5-hydroxyproline (5-Hyp), and with 5-Hyp to finally produce Glutamic acid [19,20]. In a recent work, we explored the reactions involving ˙OH attack to the different C atoms of Pro to evaluate the formation of 5-Hyp, using a theoretical approach, and suggested that the formation of 5-Hyp is unlikely to occur [21]. We predicted that the reaction should always occur on the carboxylate face (*s-face*) of Pro, to produce either 3,4-Δ-Pro or pyrroline-5-carboxylate (P5C) [21].

In this scenario, the present work aims to evaluate the •OH-attack on the N atom to assess the competitiveness of this pathway as opposed to those described on the C atoms [21]. We found out that •OH-attack on the N atom of Pro is competitive with the most favored ones over the C atoms, and can lead to the formation of γ-aminobutyric acid (GABA), which was also reported to accumulate in response to abiotic and biotic stress [22].

Methods

Molecular systems

We explored H-atom abstractions by •OH from the 4-endo and 5-endo conformer of the zwitterionic form of Pro in aqueous solution. In the 4-endo conformation the C2, C3, C5 and N atoms define almost a plane, while in the 5-endo conformer the C2, C3, C4 and N atoms are almost co-planer. As shown in the Fig. 1, the primary step in the reaction of •OH with Pro (Step 1) is characterized by the H-atom abstraction by radical attack to the N atom at both faces of the pyrrolidine ring (i.e. from the side of the carboxylate group or from the opposite one, respectively labeled s/o faces). At Step 2 the release of the carboxylic group as CO_2 leads to the formation of the radical pyrrolidin-1-yl (Pyr•). The Pyr• can react again with •OH -or with other molecules able to abstract hydrogen atom from NH group- to yield delta-1-pyrroline (Δ^1-Pyr) and water. Alternatively the Pyr• can react with H_2O_2 molecule and produce two closed shell molecules: Pyrrolidine (Pyr), and the acid form of superoxide.

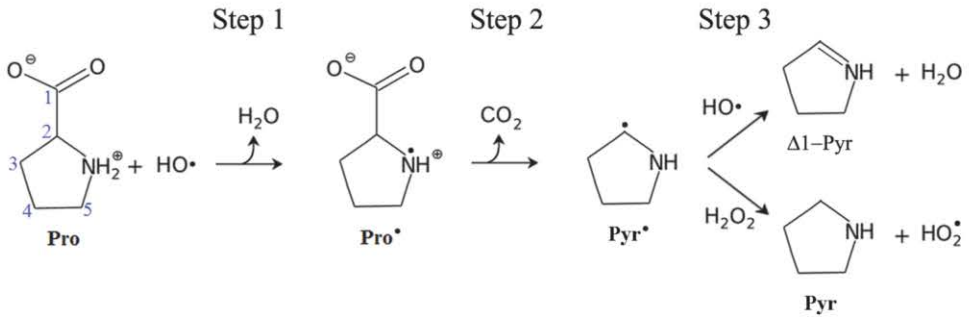

Fig 1. Reactions pathways of Pro zwitterion with $^{\bullet}OH$ in aqueous solution. Atom numbering scheme is shown over Pro in blue numbers.

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Level of theory

The structures of each stable species (reactants, products, pre-reactive intermediate complexes (IC), and transition states (TS)) were fully optimized in aqueous solution at the (U)M06/6-31G (d,p) level [23] coupled with the IEF-PCM polarizable continuum model [24–26]. Calculations were performed without imposing symmetry restrictions and using solute cavities adapted to the molecular shape and built with Bondi *radii* [27]. An ultrafine pruned grid, having 99 radial shells and 590 angular points per shell was employed for numerical integration in all M06 calculations in order to reduce possible errors in calculating energies and barriers [28]. Expectation values of the spin operator S^2 were checked to be below 0.76 and 2.01 for doublet and triplet open-shell species, respectively, to minimize spin contamination. The nature of each stationary point was carefully verified by inspection of the eigenvalues of the analytic Hessian in aqueous solution. Thermochemistry was evaluated at 298.15 K relying on the standard treatment for assessing thermal contributions (rigid rotor, harmonic vibrations with no scale factor, etc.) as implemented in the Gaussian09 program [29]. Non-electrostatic contributions (cavitation, dispersion and repulsion) [30,31] to the solvent free energy were also evaluated at 298.15 K. The reaction coordinate of each transition state (TS) was visually inspected by animation of the eigenvector associated to the imaginary frequency. IRC minimum energy reaction paths [32] towards reactants or products were generated with the HPC algorithm [33] including 45 steps for each side with a step size of 2 Bohr/amu$^{1/2}$. All the representative structures obtained from each side of the reaction path, were thus used as the starting point for optimizing the structure of the corresponding intermediate complexes (IC).

The Natural Bond Orbital (NBO) [34,35] population analysis was performed to evaluate the bonding characteristics between the C_1-C_2 atoms of reactants, TS, I_C and products. The orthogonal set of localized orbitals obtained through this analysis were also used to compute the Wiberg bond index for the decarboxylation process [36,37].

Single-point calculations at the MP2 frozen core level (using the 6-31G(d,p) basis set) were performed to analyze the spin and electron densities of all the characterized structures. The Atoms in Molecules (AIM) approach [38] was used to follow the spontaneous decarboxylation process, finding the bond critical points between the atoms in the plane of the carboxyl group in Pro.

All the calculations were performed using Gaussian09, rev. A.1 or B.1 [29], while the molecular drawings were built using either Gaussview 5 or VMD 1.9.1 [39]. The AIM analysis was achieved with the AIM-UC program [40].

Results and Discussion

The amine group of Pro is one of the most favored reaction sites for H-abstraction by the hydroxyl radical

Four different reaction pathways for the •OH-attack to the N atom of Pro zwitterion were evaluated. These pathways include, for both 4-endo and 5-endo conformers of Pro, the attack either by the side of the carboxyl group (*s-face*), or its opposite (*o-face*). The corresponding energetic barriers (Table 1) evaluate the competitiveness of these pathways as opposed to those recently described by us [21] on the C atoms. The lowest barriers in terms of free energies for each site of attack are represented in Fig. 2. Remarkably, after the H-abstraction occurs on the N atom of 5-endo Pro, the carboxyl group is destabilized and dissociated from the ring (Fig. 3). The barrier of these reactions are small, lowering when the attack occurs from the *s-face*. Moreover, the barriers related to the *s-face* abstraction over N are lower ∼by 1 kcal/mol than those previously described for the most favorable H-abstractions on C3/C4 atoms (Table 1), all of them near to the diffusion-controlled limit. Pre-reactive complexes, obtained from the ends of the intrinsic reaction coordinate path, are found to be stable in terms of enthalpy (6–7 and 2–3 kcal mol^{-1} for the *s*- and *o*-face respectively). However in terms of free energy these pre-reactive complexes turn unstable due to unfavorable entropic contributions from 2 to 6 kcal mol^{-1}. These results would eventually preclude the possibility of these intermediating complexes to act as kinetic traps, as it was found for other H-abstraction by •OH from Leucine dipeptide [41].

Hydroxyl attack at the N atom triggers Pro decarboxylation

Taking into account that the 4-endo and 5-endo forms of Pro exist in solution in equal proportions [42], and that the lower energy barriers were found for those species when the attack occurs on the *s*-face, we focused on the s-face reaction pathway for both species to evaluate in detail the structural changes that produce the release of carboxyl group from the pyrrolidine ring. Fig. 3 shows the structure of reactants, intermediate complexes, transition state and products for this reaction.

Table 1. Relative energies (in kcal mol^{-1}) for the species involved in Steps 1 in terms of enthalpies (ΔH) and free energies (ΔG) in aqueous solution at 298.15 K.

Site/face	Unimolecular barrier		Bimolecular barrier		Reaction energy	
	ΔH	ΔG	ΔH	ΔG	ΔH	ΔG
Ns 4-endo	−0.30	−0.18	−6.4	3.0	−28.2	−30.8
No 4-endo	3.0	3.9	0.58	8.9		
Ns 5-endo	−0.30	0.73	−7.0	3.1	−34.1	−32.0
No 5-endo	3.3	2.7	−0.09	8.7		
Reference values for C atoms taken from reference [21]						
C2 4-endo	1.1	1.2	−1.5	6.9	−20.1	−22.3
C3s 4-endo	1.7	2.0	−5.1	4.6	−20.1	−22.6
C3o 4-endo	−0.9	−0.3	−3.0	6.2		
C4s 4-endo	1.6	1.5	−5.1	4.1	−20.1	−22.8
C4o 4-endo	1.0	2.3	0.0	8.9		
C5s 4-endo	3.9	5.2	−3.3	6.7	−18.5	−20.7
C5o 4-endo	1.3	0.9	−0.9	7.3		

doi:10.1371/journal.pone.0115349.t001

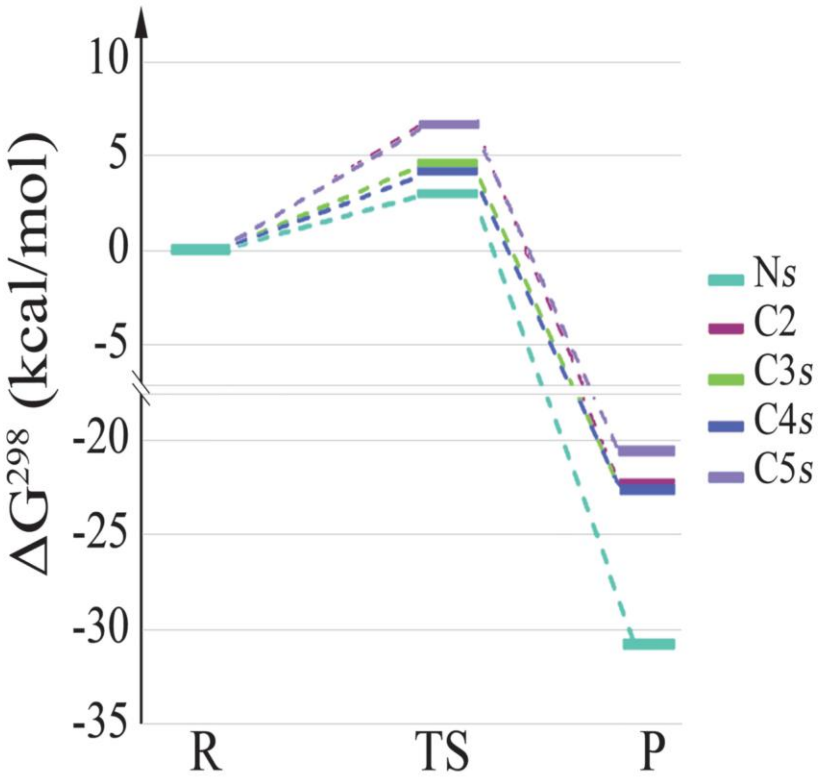

Fig 2. Energy profiles for the most favorable reactions according to Table 1. Free energies (in kcal mol^{-1}) relative to the reactants were computed in aqueous solution at 298.15 K. Energies for the H-abstraction at the C atoms were taken from [21].

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Table 2 shows the main structural parameters of the species involved in the Step 1 on *s-face*. Once the abstraction reaction has reached the TS, the distance between the C_α and the carboxyl group (C_α-COO^-) increases from 1.529 (TS) to 1.795 Å (Product), in the 4-endo conformation, and from 1.528 (TS) to 2.711 Å (Product) in the case of the 5-endo conformer. This reveals a

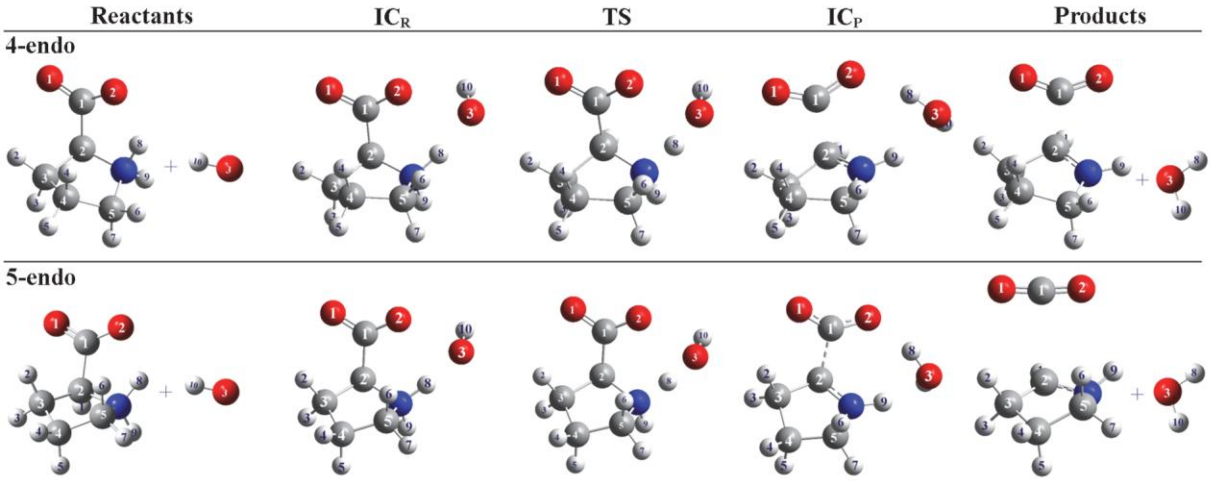

Fig 3. Structures involved in the H-abstraction by $^{\bullet}OH$ from N atom of the 4-endo and 5-endo-Pro. Note that IC_R and IC_P are the intermediary complexes for the reactants and products side respectively.

doi:10.1371/journal.pone.0115349.g003

Table 2. Selection of main structural parameters involved in Steps 1.

	4-endo conformation					5-endo conformation				
	Reactive	**IC_R**	**TS**	**IC_P**	**Product**	**Reactive**	**IC_R**	**TS**	**IC_P**	**Product**
Distances (angstroms)										
C_1-C_2	1.546	1.536	1.529	**1.722**[a]	**1.795**	1.543	1.535	1.528	**1.704**	**2.711**
C_2-N	1.510	1.494	1.481	**1.382**	1.373	1.505	1.495	1.482	**1.384**	1.397
N-H_8	1.045	1.051	**1.207**	**2.709**	-	1.040	1.052	**1.202**	**2.696**	-
C_1-O_1	1.243	1.241	**1.235**	1.222	1.219	1.243	1.241	**1.236**	**1.223**	**1.168**
C_1-O_2	1.258	1.267	1.281	**1.231**	1.218	1.258	1.267	1.280	**1.233**	**1.168**
O_2-H_8	1.808	**2.329**	**2.287**	**1.825**	-	1.847	**2.186**	**2.284**	**1.786**	-
O_3-H_8	-	1.670	**1.278**	**0.978**	-	-	1.668	**1.285**	**0.980**	-
Angles (degrees)										
C_2-C_1-O_2	115.8	**119.2**	120.3	**112.2**	109.4	115.6	**119.6**	120.6	**113.5**	**92.1**
O_1-C_1-O_2	128.9	125.5	122.0	**135.4**	**139.9**	128.9	125.3	122.0	**134.6**	**173.2**
N-C_2-H_1	109.4	109.0	109.5	**113.4**	115.2	108.1	108.0	108.7	112.4	**119.1**
N-H_8-O_3	-	163.4	**168.4**	**85.6**	-	-	162.1	**168.0**	**86.5**	-
Dihedral (degrees)										
H_8-N-C_1-O_2	2.7	**26.4**	**19.1**	**5.1**	-	–8.7	**23.9**	**12.4**	8.7	-

[a] Note that major changes are highlighted in bold.

doi:10.1371/journal.pone.0115349.t002

weakening of the C_1-C_2 bond, while the shortening of the C_2-N distance, in both conformations, is characteristic of a double bond (Table 2). On the other hand, the angle O_1-C_1-O_2 of the carboxyl group becomes less acute (particularly in the 5-endo conformer) and the C = O distances of these groups become shorter, resembling to the CO_2 molecule. When the abstraction occurred from 5-endo Pro no minima was found on the product side until a complete dissociation of the carboxyl group occurs (the final C_1-C_2 distance is 2.711 Å, and the value of the O_1-C_1-O_2 angle is 173.4°). These results provide strong evidence that the $^{\bullet}$OH-attack on the N site of Pro might trigger its decarboxylation, giving the formation of pyrrolidin-1-yl (Pyr$^{\bullet}$). The Pro decarboxylation has also been evidenced experimentally *in vitro* in presence of oxidative species, such as permanganate [43] and copper [44]. In the same way, Bonifacic *et. al.* [45] studied the reaction between $^{\bullet}$OH and Gly finding that $^{\bullet}$OH attacks occur exclusively on the NH_2 group, leading to the formation of $HN^{\bullet}$-CH_2-CO_2^- and $^+H_2N^{\bullet}$-CH_2-CO_2^-. The latter compound decompose (on the nanosecond time scale) to CO_2 and H_2N-$^{\bullet}CH_2$ [45].

Table 3. Wiberg bond index for the C_1-C_2 bond of the 4-endo and 5-endo conformations of proline along the step 1.

	C_1-C_2 bond in 4-endo conformation				
	Reactive	**IC_R**	**TS**	**IC_P**	**Product**
Wiberg bond index	0.9117	0.9245	0.9379	0.6385	0.5529
% relative to reactive	100	101	103	70	**61**
	C_1-C_2 bond in 5-endo conformation				
	Reactive	IC_R	TS	IC_P	Product
Wiberg bond index	0.9147	0.9292	0.9434	0.6657	0.0441
% relative to reactive	100	102	103	73	**5**

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To further characterize steps 1 and 2 (see Fig. 1), the H-abstraction and the *a posteriori* decarboxylation were analyzed using the Wiberg indices, spin densities (the difference between the alpha and beta densities) and the AIM approach.

In Table 3 we present the Wiberg index for the C_1-C_2 bond in both 4-endo and 5-endo conformations along the structures of step 1. As shown, the results are in agreement with the geometrical parameters (Table 2). The C_1-C_2 bond is slightly strengthened up to the TS, and then quickly weakens being almost completely broken in the product of the 5-endo. In both conformers we exhaustively search for a TS on the decarboxylation process, but all the attempts led to a quick release of the CO_2 group, suggesting that step 2 is barrierless.

The spin density is a good quantum descriptor to study the unpaired electron along the reaction path. As shown in Fig. 4, the unpaired density moves from being delocalized over the oxygen atoms (see IC_R and TS in Fig. 4) to be localized over the C_2/Cα and N atoms in the products (see IC_P and Products). During the H-abstraction, the spin density moves from the oxygen atoms (mainly from $^{\bullet}$OH), through the N atom (forming a cationic radical), to the C_2/Cα atom, leading to the decarboxylation of the Pro and the strengthening of the Cα-N bond (see Table 2). The final result is the spontaneous formation of a Pyr$^{\bullet}$ and carbon dioxide (clear in the case of the 5-endo conformer), in a barrierless reaction, for which no activation energy is required and no TS can be defined. It is worth mentioning that the decarboxylation occurs differently in the 4-endo and 5-endo forms. The decarboxylation seems to be well defined in the 5-endo. In the 4-endo reaction the spin density does not show a significant differences between IC_P and the product (Fig. 4), although, as mentioned before, the C_1-C_2 distance slightly increases between these two stable species (see Table 2), suggesting that we found a stable complex, in the product side of the H-abstraction, before decarboxylation (in agreement with the Wiberg indices reported in Table 3). In the latter case, also the AIM analysis reveals the presence of a bond critical point (bcp), suggesting that there still is a covalent bond connecting the carboxyl group to the pyrrolidine ring. This bcp is not present in the product of the 5-endo reaction where the decarboxylation is evident. In the 4-endo form, the Pyr$^{\bullet}$ and the CO_2 molecules seem to be trapped in a local minimum forming a stable complex, which could explain in part the low rate of decomposition observed experimentally [45].

Complementary reactions to obtain closed-shell species

The Pyr$^{\bullet}$ can react with different molecules to produce closed shell molecules. Here we suggest two different mechanisms: one involves another H-abstraction by $^{\bullet}$OH from the N atom of the pyrrolidine ring; and the other one involves the H-abstraction by Pyr$^{\bullet}$ from hydrogen peroxide (see Fig. 1). In both cases the reaction leads to the formation of Δ^1-Pyrroline (Δ^1-Pyr). Note that after decarboxylation it is not possible to define the s- and o-face, and for this reason there is only one possible conformation in the two reactions explored in Step 3 (Fig. 5).

As expected (in Step 3) the reaction with $^{\bullet}$OH has a lower energy barrier as well as being more exothermic than the reaction with H_2O_2 (see Table 4). Both proposed mechanisms are likely to occur, and while Step 3a is irreversible, Step 3b can occur in both directions. Once again the intermediate complexes are more stable in terms of enthalpies, but not anymore when entropic contributions are considered.

Connecting Pro to GABA

In plants, Δ^1-Pyr produced in Step 3a is the substrate of pyrroline dehydrogenase (PYRR-DH) which converts it into γ-aminobutyric acid (GABA) [46]. GABA is well-documented to accumulate under stress conditions [47]. For this reason, several protective roles had been assigned to GABA, such as the contribution to the C:N balance, the regulation of cytosolic pH, the

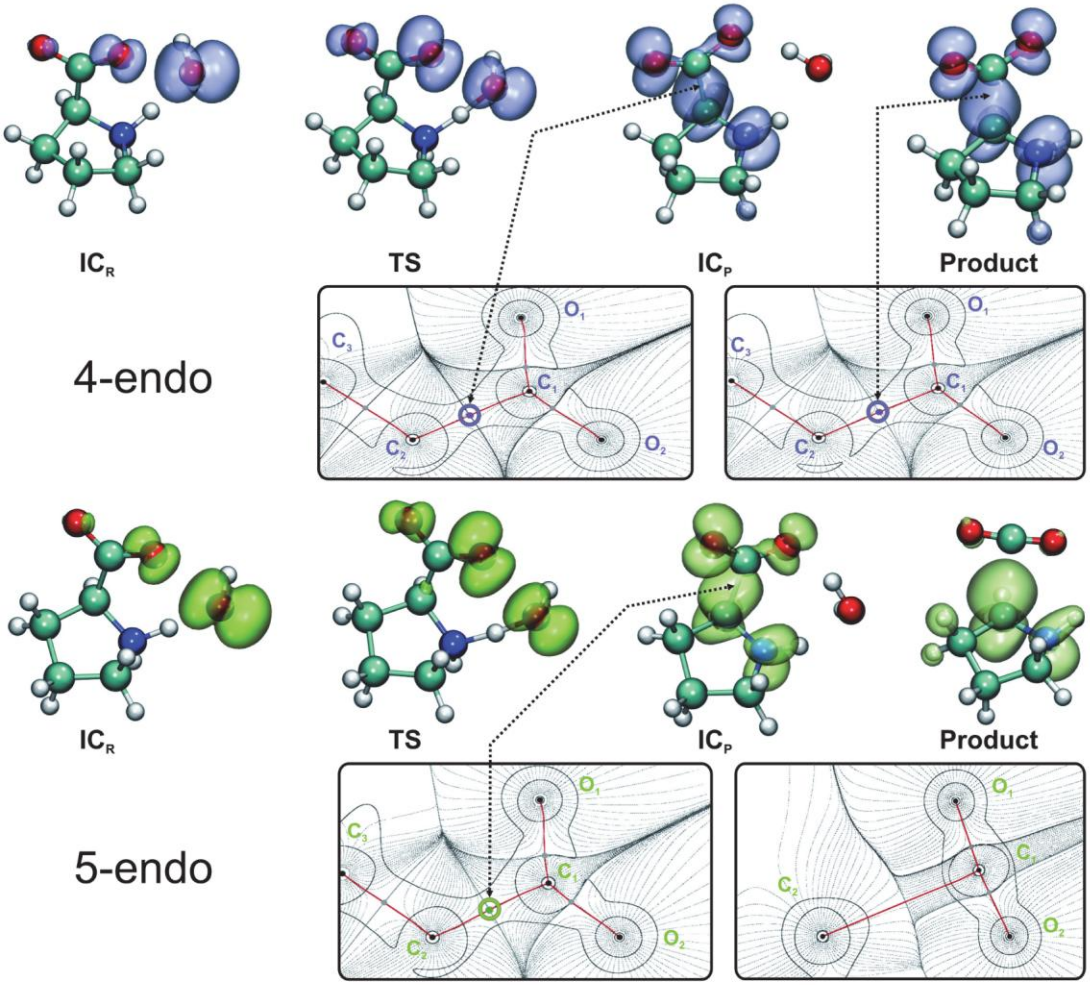

Fig 4. Spin densities and AIM analysis. For the sake of comparison, the same isosurface of value 0.003 |e|/Å^3 representing the difference between the alpha and beta electron densities was depicted in violet or green, for the 4-endo and 5-endo forms respectively. For the products and the intermediate species on the product side (IC$_P$), the presence of bond critical points (bcp) was evidenced by means of AIM. The bcp connecting the carboxyl group to the pyrrolidine ring is depicted in violet or green, whereas the remainder bcps are shown in gray. The nuclear critical points (located at the position of the nuclei), the basin paths, and the gradient field are depicted in solid or dashed black lines. The bond paths, defined by the chosen 2D projection (plane), are shown in red.

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protection against oxidative stress, the defense against insects, the osmoregulation, and cell signaling [47]. Most of these protective roles have also been attributed to Pro [11]. The principal way in which GABA is synthetized is from glutamic acid, by the enzyme glutamate decarboxylase [22], while a secondary pathway involves polyamines and the formation of Δ^1-Pyr [46,48]. For example, the CMSII mutant of *Nicotiana sylvestris* has reduced glutamate decarboxylase (GAD) activity, but the GABA content nevertheless increased more than two- fold from the base level when treated with NaCl [49], suggesting that the accumulation of GABA could be mediated by polyamines oxidation. This pathway requires the activity of pyrroline (γ-aminobutyraldehyde) dehydrogenase, an enzyme not produced in stress conditions [48], meaning that GABA formation should be mediated by the accumulation of GABA precursors. Here we propose an alternative non-enzymatic pathway (shown in blue in Fig. 6) in which Pro can contribute to the formation of the GABA precursor Δ^1-Pyr, giving a possible explanation to connect the simultaneous accumulation of Pro and GABA that occur in stress conditions. We

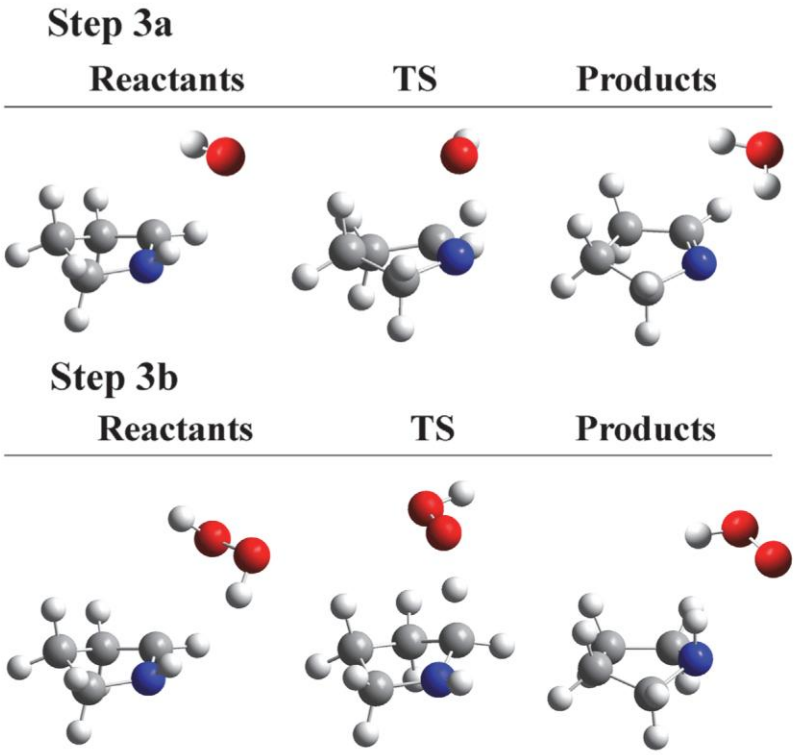

Fig 5. Structures involved in Step 3. In Step 3a, the H-abstraction from Pyr$^{\bullet}$ at the N atom by $^{\bullet}$OH produces Δ^1-Pyr. In Step 3b, the H-abstraction from H_2O_2 by Pyr$^{\bullet}$ produces Pyr.

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recently demonstrated that a plastidic glutamine synthetase mutant of *Lotus japonicus* with unaltered glutamate content but lower proline content, had a lower accumulation of GABA in response to osmotic stress when compared with the wt [50].

Biological significance of the reaction of Pro with $^{\bullet}$OH in plants under stress

In the chloroplasts, the co-localization of Pro, $^{\bullet}$OH and H_2O_2 is essential for the proceeding of the reactions described in Steps 1 and 3. In these organelles, the electron leakage of O_2 is the major source of $O_2^{\bullet -}$ [51], which leads to the formation of H_2O_2 by SOD activity in a diffusion-limited reaction. $^{\bullet}$OH can be produced from H_2O_2 by Fenton's reaction or by homolytic cleavage under UV exposure. In addition, Pro reaches concentrations of at least 160 mM [52]

Table 4. Relative energies (in kcal mol^{-1}) for the species involved in Steps 3 in terms of enthalpies (ΔH) and free energies (ΔG) in aqueous solution at 298.15 K.

Step	Site of attack	Attacked by	Unimolecular barrier		Bimolecular Barrier		Reaction Energy	
			ΔH	ΔG	ΔH	ΔG	ΔH	ΔG
3a	N	$^{\bullet}$OH	4.4	5.1	–1.8	8.1	-88.7	-88.7
3b	C_2	H_2O_2	5.4	6.6	–6.4	11.7	-5.1	-4.2

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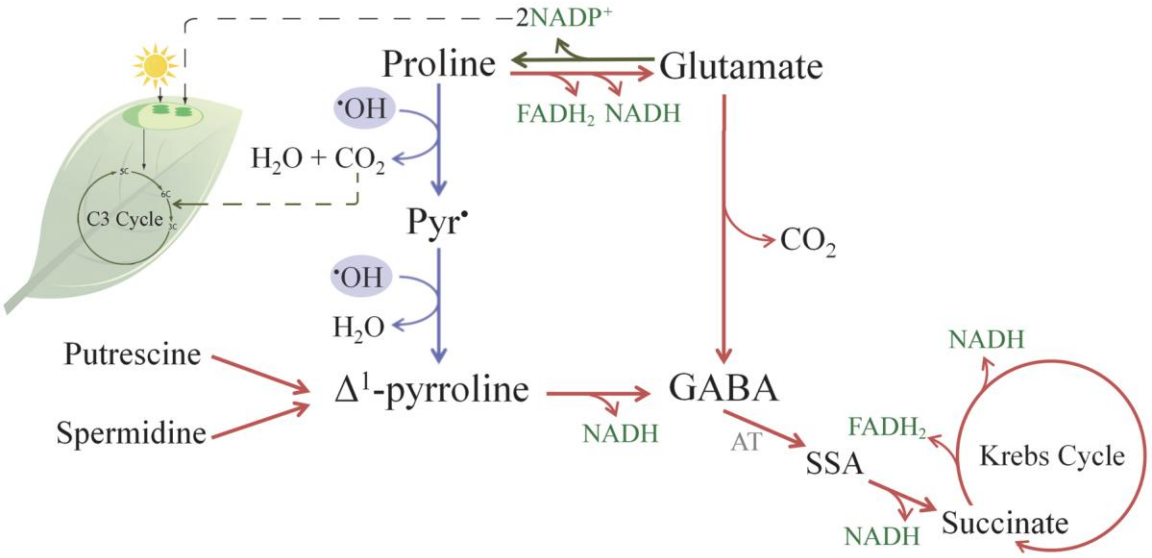

Fig 6. Suggested pathway to connect Pro and GABA and its possible implications in major metabolic processes. Blue lines represent the non-enzymatic reactions proposed to connect Pro and Δ^1-Pyr by $^{\bullet}OH$ scavenging. In the non-enzymatic reactions two $^{\bullet}OH$ are captured and the CO_2 released could enter the C3 cycle. Red and green lines represent catabolic and anabolic pathways respectively. SSA stands for succinic semialdehyde, and AT stands for amino transferases.

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(even in non-stressful condition) into these organelles becoming more abundant than every common antioxidant (i.e.: 0.8–2.4 mM for glutathione; 12–25 mM for ascorbate) [53].

Besides the H_2O_2 production in chloroplasts, H_2O_2 is also generated by photorespiration in the peroxisomes [54]. Other H_2O_2 sources are the copper-containing oxidases and peroxidases, being the latter the main responsible for H_2O_2 production during oxidative burst in stress conditions in plants [54]. Since H_2O_2 has a long lifetime and can diffuse through organelles [55], it can be assumed to be available in all the cell to yield $^{\bullet}OH$ or react with $Pyr^{\bullet}$.

Because of the main co-localization of ROS and Pro in chloroplasts, the CO_2 released from Pro accumulates predominantly in these organelles. There, the photosynthetic machinery is able to use CO_2 by fixation through the C3 Cycle (see Fig. 6). Despite being a hypothesis, that will require further experimental confirmations, this localized production of CO_2 could be an advantage for plants under stress conditions (i.e. drought). In such conditions the stomata are close to avoid water loss, limiting the uptake of CO_2, that leads to a reduction in carbon fixation and the accumulation of reducing power (NADPH). All these combined features will most probably produce ROS by electron leakage. Therefore, it is not unreasonable to think that the CO_2 produced during Pro reactions would help reducing the accumulation of NADPH and the generation of ROS.

Pro synthesis in green tissues was proposed to regenerate $NADP^+$, helping in the maintenance of an adequate $NADP^+$/NADPH ratio inside the cells [56], and that Pro could translocate to the roots to be catabolized [56]. In addition to the NADPH consumption produced during Pro synthesis, our work suggests that Pro could also help to reduce the $NADP^+$/NADPH ratio, by releasing CO_2 that will enter the C3 cycle and consume NADPH.

On the other hand, it is known that transaminases turn GABA into succinic semialdehyde (SSA) that is then converted to succinate by succinic semialdehyde dehydrogenases (Fig. 6) [57,58]. These reactions, known as GABA shunts, produce substrates of the mitochondrial respiratory chain (succinate and NADH), which ultimately generates ATP [47]. GABA shunts affect the redox balance in the cell, because succinate enters the Krebs Cycle bypassing three sites

of NADH production, thus reducing the NADH/NAD^+ ratio [49,59,60]. This reduction in NADH/NAD^+ ratio activates key enzymes of the Krebs cycle [61]. Additionally, it is known that the succinyl-CoA ligase and the α-ketoglutarate dehydrogenase enzymes are degraded under oxidative stress, limiting the ATP production in the Krebs cycle [62]. GABA shunts assure the production of ATP even in stressed conditions, reason why GABA shunts were considered a protection against oxidative stress [47].

Overall, we consider that the non-enzymatic connection between Pro and GABA presented in this work is a reasonable alternative to catabolize Pro obtaining reducing power, ATP, carbon and nitrogen, even in stressed conditions when the catabolic enzymes of Pro are inactivated. This beneficial aspects of Pro and the protective role against $^{\bullet}OH$ are in line with the multifunctional role that has been assumed for Pro.

Conclusions

Hydrogen abstraction from the N atom of Pro by $^{\bullet}OH$ radical produces the decarboxylation of Pro, and leads to the formation of $Pyr^{\bullet}$. This reaction mechanism, proposed herein for the first time, is competitive as opposed to those described previously for the C atoms [21]. These theoretical results are in line with experimental data about amino acid decarboxylation under $^{\bullet}OH$ attacks. The Δ^1-Pyr produced by H-abstraction from $Pyr^{\bullet}$ would contribute to the production of GABA under PYRR-DH catalysis, an essential molecule for plants, that also accumulates in stress conditions. Thereby, we proposed an alternative way to synthetize GABA, through a non-enzymatic reaction relevant in plants under oxidative stress. Finally, this work highlights a new beneficial characteristic of Pro accumulation, as the contribution to maintain the photosynthetic activity in stressed plants.

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Author Contributions

Conceived and designed the experiments: SS PDD ELC. Performed the experiments: SS PDD. Analyzed the data: SS PDD ELC. Contributed reagents/materials/analysis tools: SS PDD ELC OB JM. Wrote the paper: SS PDD ELC OB JM. Contributed the biological background of the problem: SS OB JM.

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IV. La prolina en la protección frente al daño nitrosativo

Cada año se publican trabajos que hacen referencia a la prolina como antioxidante. En muchos de ellos se la considera como un antioxidante no enzimático de bajo peso molecular, en la misma categoría que el glutatión y el ácido ascórbico (Bandurska et al., 2013; Dinakar et al., 2009; Erdal, 2012; Filippou et al., 2014; Huang et al., 2014; Iqbal et al., 2014; Sekmen et al., 2014; Semida and Rady, 2014; Wilson et al., 2014). En un trabajo publicado en PNAS Chen and Dickman (2005) proponen que la prolina es un potente antioxidante. Sin embargo el hecho de que la prolina no reaccione con el $^1O_2$, una especie más reactiva que la mayoría de ROS/RNS, pone en cuestionamiento dicha capacidad antioxidante. Además también hay evidencias de que la prolina tiene poca reactividad con el peroxinitrito (Alvarez et al., 1999), pero estas evidencias no han sido consideradas en el campo de las plantas.

En el artículo que se presenta a continuación, que fue enviado para su publicación en *Annals of Botany*, se puso en evidencia que la prolina no protege frente al $O_2^{\cdot-}$, $^{\cdot}NO$, $ONOO^-$ y $^{\cdot}NO_2$. Estos resultados sumados a los del $^1O_2$, nos permiten demostrar que el rol antioxidante que se le asigna a la prolina es marginal y sólo se limita a la protección frente al $^{\cdot}OH$.

De todas formas no descarta que la acumulación de prolina pueda contribuir en la protección frente al daño oxidativo, algo que está propuesto en muchos trabajos.

En el trabajo se busca aclarar que este efecto es poco probable que se deba a un rol antioxidante directo y se discuten distintos mecanismos por los cuales la acumulación de prolina contribuye a aliviar el daño oxidativo.

Type of article: Research in context

Title: Proline is not directly involved in protection against nitro-oxidative damage mediated by superoxide, nitric oxide and peroxynitrite

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Running Title: Proline is not directly involved in protection against $O_2^{\bullet-}$, $^{\bullet}NO$ and $ONOO^-$

Abstract

- Background and Aims: Proline (Pro) is accumulated in plants exposed to diverse types of stresses. In many of these stresses there is a nitro-oxidative component and for that reason, Pro has been considered to have antioxidant properties. This study evaluates whether Pro has the capacity to protect proteins against peroxynitrite, a molecule with a strong oxidant capacity which can mediate protein nitration.
- Methods: *Lotus japonicus* leaf samples were exposed to SIN-1 (3-morpholinosydnonimine), as peroxynitrite donor, in the presence and absence of 100 mM Pro. We measured the activity of NADP-dependent isocitrate dehydrogenase (NADP-ICDH), an identified protein susceptible to be nitrated, and the protein nitration profile.
- Key Results: The presence of 100 mM Pro did not prevent enzymatic activity decay, suggesting that Pro did not protect against peroxynitrite. The protein nitration profile was not modified in presence of 100 mM Pro. However, the presence of 1 mM urate, a peroxynitrite scavenger, prevented protein nitration evidenced by NADP-ICDH activity and protein nitration profile.
- Conclusion: Pro is not an efficient scavenger of peroxynitrite ($ONOO^-$), superoxide ($O_2^{\bullet-}$), nitric oxide ($^{\bullet}NO$) and nitrogen dioxide ($^{\bullet}NO_2$), revealing that this amino acid cannot protect against many ROS/RNS as has been extensively proposed.

Keywords

Antioxidant; scavenger; ROS; RNS; plant stress; proline; nitro-oxidative stress; superoxide; nitric oxide; peroxynitrite

Introduction

Plants subjected to environmental stresses overproduce reactive oxygen species (ROS) and a family of nitric oxide ($^{\bullet}NO$) derived molecules, called reactive nitrogen species (RNS). When this overproduction occurs, nitro-oxidative damage and programmed cell death takes place (Corpas and Barroso, 2013; Serrano et al., 2012). In particular, $^{\bullet}NO$ and superoxide ($O_2^{\bullet-}$) are rapidly combined to form peroxynitrite which is a potent oxidant molecule. In biological conditions the anionic form of peroxynitrite ($ONOO^-$) coexists with its conjugated acid form (ONOOH), with a p*K*a~ 6.8 (Carballal et al., 2014). Usually the term peroxynitrite refers to both peroxynitrite anion ($ONOO^-$) and peroxynitrous acid (ONOOH) (Radi, 2012). Peroxynitrous acid spontaneously produces hydroxyl radical ($^{\bullet}OH$) and nitrogen dioxide ($^{\bullet}NO_2$) by homolysis (Beckman et al., 1990). The presence of peroxynitrite in plant cells leads to protein nitration, by modification of tyrosine (Tyr) residues into 3-nitrotyrosine (Corpas et al., 2007), and nitration of unsaturated lipids producing nitroalkenes (Fazzari et al., 2014). On the one hand, it was demonstrated that protein nitration, developed in plants exposed to diverse abiotic/biotic stresses, can lead to the inactivation of specific proteins such as ferredoxin-NADP reductase (Chaki et al., 2011a), carbonic anhydrase (Chaki et al., 2013), NADP-isocitrate dehydrogenase (NADP-ICDH) (Begara-Morales et al., 2013), NADH hydroxypyruvate reductase (Corpas and Barroso, 2013), superoxide dismutase (Mn- and CuZn-SOD) (Holzmeister et al. in press) and ascorbate peroxidase (Begara-Morales et al., 2014).

On the other hand, plants accumulate proline (Pro) in response to many stress conditions, such as salinity, drought, heavy metals or UV-radiation, among others, reaching a concentration of over 160 mM (Barnnet and Naylor, 1966; Büssis and Heineke, 1998; Szabados and Savouré, 2010). Several hypotheses have been proposed to explain the role of Pro under stress (Dinakar et al., 2009; Kavi Kishor and Sreenivasulu, 2014; Rejeb et al., 2014; Szabados and Savouré, 2010). Some of them were put forward trying to find a link between Pro and ROS/RNS as both are produced upon exposure to stress conditions. Along that line of thought, it was

hypothesized that Pro acts as an antioxidant which could protect biomolecules from oxidative damage. Nowadays, it is known that some reactive species, like H_2O_2, $O_2^{\bullet-}$ and $^{\bullet}NO$ produce accumulation of Pro by inducing the expression of Pro biosynthetic genes and repressing its catabolic genes (Rejeb et al., 2014).

Through different *in vitro* assays Pro was suggested to have antioxidant properties. In 1989, Pro was shown to be an effective $^{\bullet}OH$ scavenger by protecting against enzyme inactivation (Smirnoff and Cumbes, 1989). Later, it was proposed to have a protective role against singlet oxygen ($^1O_2$) (Alia et al., 2001, 1991). Then through an *in vivo* assay, Pro was proposed to have a potent antioxidant activity in yeast cells (Chen and Dickman, 2005). Using Arabidopsis knockout mutants for *p5cs1*, the osmotic inducible gene of Pro biosynthesis, it was observed that the imposition of salt stress produces greater accumulation of H_2O_2 and lipid peroxidation than that observed in wt plants (Szekely et al., 2008). These kinds of works raise the following question: Is Pro playing a role as an antioxidant by reacting with reactive species or through the protection or induction of detoxifying enzymes during stress? None of these properties have been unequivocally demonstrated yet (Trovato et al. 2008; Szabados and Savouré 2010; Signorelli et al. 2013*a*). Moreover, in case Pro acts as an antioxidant, why is there limited information about the reactivity of Pro and reactive species? Nevertheless, far from being questioned, every year many research papers consider or refer to Pro as a non-enzymatic low molecular weight antioxidant, in the same category as glutathione and ascorbic acid (Bandurska et al., 2013; Erdal, 2012; Huang et al., 2014; Semida and Rady, 2014; Wilson et al., 2014).

Because of the absence of new and direct evidence on the protective role of Pro against reactive species and the mechanisms of reaction, very recently we evaluated the mechanisms in which Pro reacts with $^{\bullet}OH$ and the products formed. The results revealed that proline can contribute to the scavenging of $^{\bullet}OH$ by a Pro-Pro cycle without consumption of Pro (Signorelli et al., 2014). Also it was showed that when $^{\bullet}OH$ attack to Pro by H-abstraction from the amine group, produces the decarboxylation of Pro and the product formed could contribute to the accumulation of GABA (Signorelli et al. in press). Along those lines, we also evaluated the

mechanism in which Pro could react with $^1O_2$ and surprisingly we found that Pro does not quench $^1O_2$ (Signorelli et al. 2013*a*) as was considered for many years. Taking into account that after $^{\bullet}OH$, $^1O_2$ is the second most reactive specie compared with other ROS/RNS, this finding makes the role of Pro as scavenger of other ROS/RNS less reactive than $^{\bullet}OH$ questionable.

In this work, we evaluated the potential protective role of Pro against peroxynitrite, $O_2^{\bullet-}$, $^{\bullet}NO$ and $^{\bullet}NO_2$. Our results indicate that Pro does not protect against these reactive species, suggesting that the role of Pro as a general antioxidant needs to be reconsidered.

Materials and Methods

Plant material and crude extracts

Lotus japonicus (Regel) Larsen cv. Gifu seeds were obtained from Ing. Agr. M. Rebuffo, INIA La Estanzuela, Uruguay. Plants were grown as described in Signorelli et al. (2013*b*). Leaf samples were collected and pooled from the first to the seventh expanded leaf. Then, leaves were frozen in liquid N_2 and ground in a mortar with a pestle. The powder was suspended in a homogenizing medium composed of 50 mM Tris-HCl, pH 7.4, 0.1 mM ethylene diamine tetra-acetic acid (EDTA), 5 mM dithiothreitol (DTT) and 0.2% (v/v) glycerol. Homogenates were centrifuged at 20,000 x *g* for 20 min (4°C) and supernatants were used immediately for assays. Samples were passed through a desalting NAP-10 column (General Electric®) to remove low molecular weight compounds.

Protection against protein nitration by Pro: treatments with SIN-1

The molecule SIN-1 (3-morpholinosydnonimine) being a protein-nitrating compound, has been demonstrated to generate equimolar amounts of $^{\bullet}NO$ and $O_2^{\bullet-}$, which reacts immediately to produce peroxynitrite (Daiber et al., 2004). Leaf extracts were incubated at 37 °C for 2 h with 0, 0.5 and 2.0 mM SIN-1 (Calbiochem) freshly made up before use. To evaluate the potential protecting effect of Pro, leaf samples incubated with SIN-1 were also supplemented with 100 mM Pro. As scavenger control, treated leaf samples with SIN-1 were also supplemented with 1 mM urate (Alamillo and García-Olmedo, 2001). All the samples were passed again through

desalting NAP-10 column to avoid any interference of SIN-1, Pro or urate with the following assays.

Immune-detection of protein nitration

Leaf samples (30 μg of protein) were separated by SDS-PAGE according to the method of Laemmli (Laemmli, 1970) in 12 % acrylamide slab gels (6 % for stacking). One gel was used to determine the protein load by staining with Coomassie Brilliant Blue G-250. For Western blot analysis, proteins were transferred to PVDF membranes with a semi-dry Trans-Blot cell (BioRad). Then, membranes were used in cross-reactivity assays with a commercial rabbit polyclonal antibody against 3-nitrotyrosine (SIGMA) diluted 1:3000. For immuno-detection, an affinity-purified goat anti-(rabbit IgG)-horseradish peroxidase conjugate (Bio-Rad) and an enhanced chemiluminescence kit (Clarity Western ECL, Bio-Rad) were used. For detection of chemiluminescence on the membrane a digital imager was used (scanner C-DiGit, Li-Cor).

NADP-dependent isocitrate dehydrogenase activity assay

NADP-ICDH (EC 1.1.1.42) activity was determined spectrophotometrically by recording the reduction of $NADP^+$ at 340 nm. The assay was performed with the different treatments, at 25 °C in a reaction medium containing 50 mM HEPES, pH 7.6, 2 mM $MgCl_2$, 0.8 mM $NADP^+$. The reaction was initiated by the addition of 10 mM 2R,3S-isocitrate as substrate (Leterrier et al., 2007). Protein concentration was determined using the Bio-Rad protein assay using BSA as standard.

Statistical analysis

An analysis of variance was performed with data from at least three independent experiments, and means from the results of experiments were compared using Tukey´s test at the $p \leq 0.05$ level.

Results

To evaluate whether Pro can act as antioxidant and protect against protein nitration, *L. japonicus* leaf extracts were incubated with SIN-1, as peroxynitrite donor, in presence and absence of 100 mM Pro (Fig. 1).

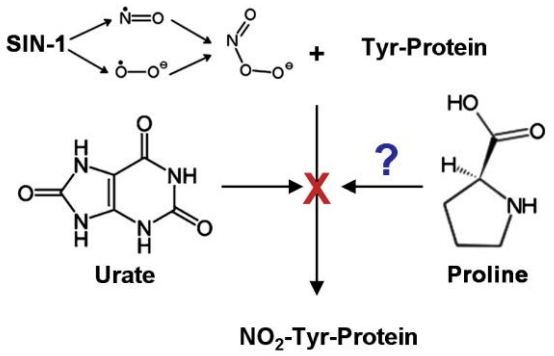

Figure 1. Hypothetical protection of Pro to protein nitration. In the scheme we summarize the experimental design to evaluate the capacity of Pro to prevent the process of protein nitration. The molecule SIN-1 (3-morpholinosydnonimine) generates equimolar amounts of $^{\bullet}NO$ and $O_2^{\bullet-}$ which react to produce peroxynitrite, a strong oxidant molecule which can mediate nitration of tyrosine residues of proteins (NO_2-Tyr-Protein). Urate is used as control because it is a recognized peroxynitrite scavenger.

Recently, the enzyme NADP-ICDH was detected to be a target of nitration and it was demonstrated that this process caused an activity inhibition (Begara-Morales et al., 2013). Therefore, this enzyme activity was measured to evaluate the potential protective effect of Pro on a specific protein. Figure 2 depicts the inhibitory effect of peroxynitrite on NADP-ICDH activity that ranges from 24 % with 0.5 mM SIN-1 to 68 % with 2 mM SIN-1. When 100 mM Pro was added activity also was inhibited, 24 % of inhibition with 0.5 mM SIN-1 and 55 % with 2 mM SIN-1. The addition of 1 mM urate, which is well-known as a peroxynitrite scavenger, was enough to prevent the inhibitory effect of SIN-1 on NADP-ICDH activity (Fig. 2).

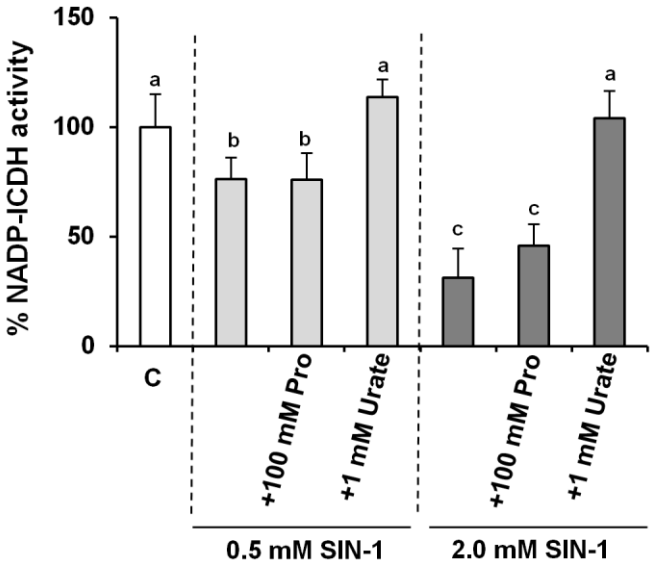

Figure 2. NADP-ICDH activity. Leaf samples were incubated with SIN-1 (0.5 or 2 mM) at 37 ºC for 2 h in the absence or presence of 100 mM Pro or 1 mM urate. The specific activity of NADP-ICDH without any treatment (C, 100% activity) was 8.2 nmol NADPH . min^{-1} . mg^{-1} protein. Data are means ± SD of at least three replicates. Same letters indicate that no statistically significant differences ($P < 0.05$).

Considering that Pro could not prevent the inactivation of NADP-ICDH activity, we evaluated the protein nitration profile to observe if this phenomenon occurred to all proteins. Because the inhibitor effects were greater at 2 mM SIN-1, we chose this concentration to perform the immunoblot analysis of leaf samples probed with an antibody against 3-nitrotyrosine. The addition of SIN-1 caused a significant increase in protein nitration (Fig. 3, upper panel) and the addition of 100 mM Pro did not reduce it (Fig. 3, upper panel). In contrast, the addition of 1 mM urate to samples treated with SIN-1 clearly showed a protective effect against protein nitration since no immunoreactive bands were detected (Fig. 3, upper panel). The protein profile indicating an equal sample loading is shown in Figure 3 (lower panel).

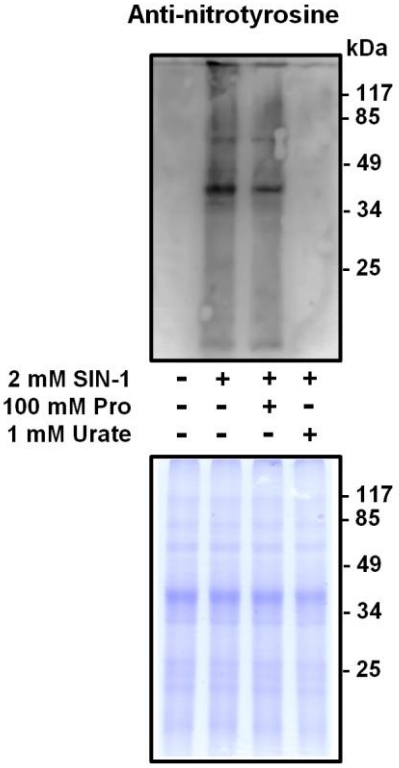

Figure 3. Immunoblot analysis of *L. japonicus* protein extract with an antibody against nitrotyrosine. Upper panel, protein nitration profile obtained by incubation of leaf protein samples with SIN-1 (peroxynitrite donor) in the presence and absence of 100 mM Pro or 1 mM urate. Lower panel, loading control of proteins stained with Coomassie Brilliant Blue G-250.

Discussion

Under certain stress conditions such as salinity or drought, higher plants can synthesize high levels of Pro in the milimolar range (Szabados and Savouré, 2010; Trovato et al., 2008). Considering that many of these stresses have a nitro-oxidative stress component, Pro has been suggested to have an antioxidant role (Bandurska et al., 2013; Dinakar et al., 2009; Erdal, 2012; Filippou et al., 2014; Huang et al., 2014; Iqbal et al., 2014; Sekmen et al., 2014; Semida and Rady, 2014; Wilson et al., 2014). We evaluated whether Pro can protect plant proteins against tyrosine nitration mediated by peroxynitrite, a NO-derived molecule which is a strong oxidant molecule (Arasimowicz-Jelonek and Floryszak-Wieczorek, 2011; Ischiropoulos, 2003; Radi, 2004).

The molecule SIN-1 (3-morpholinosydnonimine) has been demonstrated to generate equimolar amounts of $^{\bullet}NO$ and $O_2^{\bullet-}$, which reacts rapidly to produce peroxynitrite (Fig. 4) (Daiber et al., 2004). The use of SIN-1 is better considered than the direct use of peroxynitrite because it is more representative of a cellular context where $^{\bullet}NO$ and $O_2^{\bullet-}$ are present to form peroxynitrite.

Our results showed that NADP-ICDH activity was reduced by the addition of 0.5 and 2 mM SIN-1 (Fig. 2) which is in concordance with results observed for NADP-ICDH of pea (Begara-Morales et al., 2013). When the leaf sample was incubated simultaneously with SIN-1 and 100 mM Pro, the inhibitory effect of SIN-1 was not prevented (Fig. 2). As Pro could not prevent NADP-ICDH inactivation, this result suggests that Pro does not have a protective role against nitration.

Because nitration does not occur by direct reaction of peroxynitrite and proteins we will described the nitration mechanism in order to analyse the putative scenarios in which Pro could establish a relationship with ROS/RNS (Fig. 4).

The anionic form of peroxynitrite ($ONOO^-$) is in equilibrium with its conjugated acid form (ONOOH) which decays by homolytic cleavage to $^{\bullet}NO_2$ and $^{\bullet}OH$ with a rate constant of 0.9 s^{-1} (Fig. 4, *k*b) (Ferrer-Sueta and Radi, 2009). In our experimental conditions, pH 7.4 and 37°C, peroxynitrite anion will be present in a proportion of 80% (Carballal et al., 2014). This form reacts with CO_2 with a rate constant of 1.6 × 10^4 M^{-1} s^{-1} (Fig. 4, *k*c), at that temperature, and can yield $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ (Denicola et al., 1996a). The radicals $^{\bullet}NO_2$, $^{\bullet}OH$, and $CO_3^{\bullet-}$ produced from peroxynitrite can react with Tyr residues to produce the tyrosyl radical ($^{\bullet}$Tyr) whit rate constants of 3.2 × 10^5 M^{-1} s^{-1}, 6.5 × 10^8 M^{-1} s^{-1} and 4.5 × 10^7 M^{-1} s^{-1}, respectively (Fig. 4, *k*c, *k*d and *k*e) (Radi, 2012). Once $^{\bullet}$Tyr is produced through any of these pathways, a $^{\bullet}NO_2$ can be added to form 3-nitrotyrosine with a rate constant of 3.0 × 10^9 $M^{-1}s^{-1}$ (Fig. 4, *k*g) (Radi, 2012). Uric acid rapidly reacts with $^{\bullet}OH$ and $^{\bullet}NO_2$, with rate constants of 1.0 × 10^9 $M^{-1}s^{-1}$ and 1.8 × 10^7 $M^{-1}s^{-1}$, respectively (Simic and Jovanovic, 1989) and therefore it can completely inhibit protein nitration (Fig. 4, Uric acid).

Our results demonstrated large amounts of Pro (100 mM) cannot prevent protein nitration in crude extracts of *L. japonicus* leaves (Fig. 3). If Pro reacted directly with peroxynitrite it would reduce protein nitration by reducing the formation of $^{\bullet}OH$, $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ (Fig. 4, Scenario 1). Since it did not happen (Fig. 2 and 3) we conclude that Pro does not react efficiently with peroxynitrite, and this result is in accordance with *in vitro* assays which showed that Pro had poor reactivity with peroxynitrite (Alvarez et al., 1999).

Considering that Pro reacts with $^{\bullet}OH$ with a rate constant of 4.8 x 10^8 $M^{-1}s^{-1}$ (Davies, 2005), half of that of uric acid, formation of $^{\bullet}$Tyr by $^{\bullet}OH$ would be reduced, explaining the slight reduction in protein nitration observed in presence of Pro (Fig. 3). However, in case Pro does not react with $CO_3^{\bullet-}$ or $^{\bullet}NO_2$, $^{\bullet}$Tyr will be formed and a $^{\bullet}NO_2$ could be added to it, unless Pro reacts with $^{\bullet}NO_2$. Because nitration is observed in presence of Pro, we concluded that Pro does not protect against $^{\bullet}NO_2$ and so Scenario 2 of figure 4 is unlikely to happen.

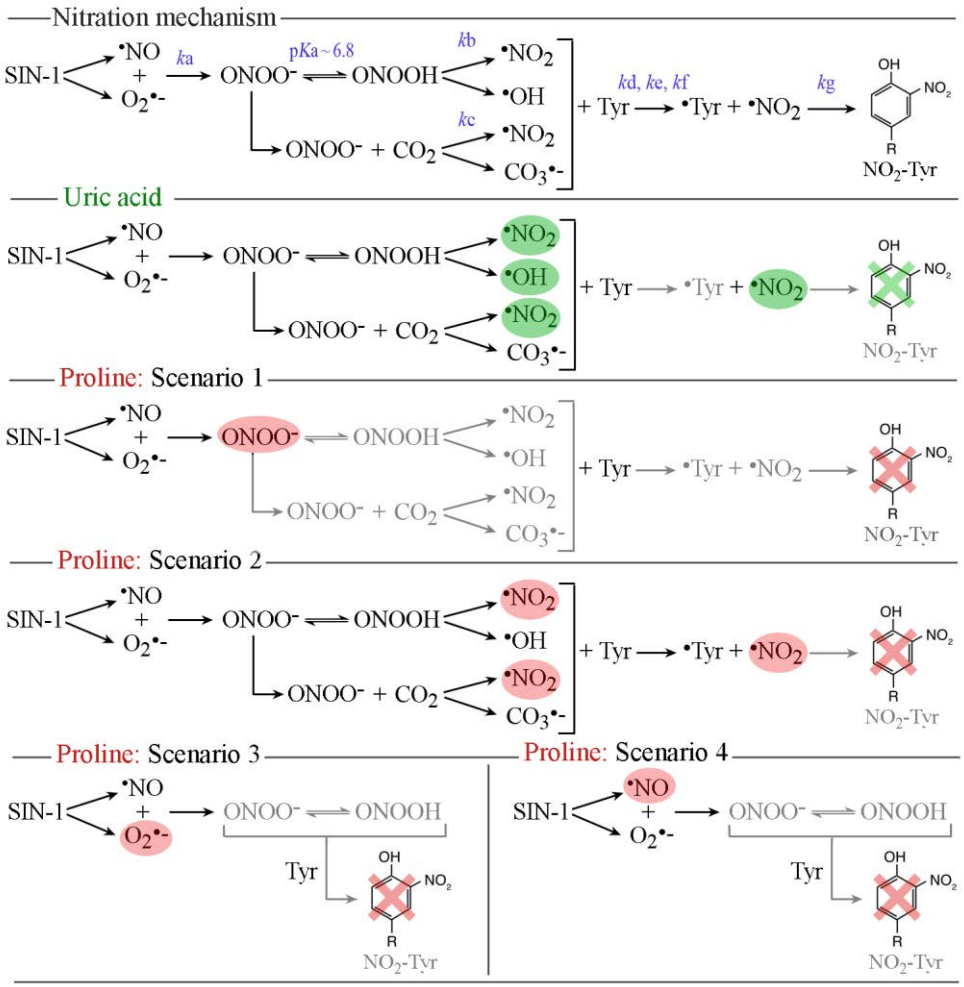

Figure 4. Putative scenarios to analyse protection against RNS/ROS by Pro. At the top we represented the Tyr nitration mechanism by SIN-1 in absence of scavengers. *k*a, 4.3 x $10^9 M^{-1}$ s^{-1}; *k*b, 0.9 s^{-1}; *k*c, 1.6 × 10^4 M^{-1} s^{-1}; *k*d, 3.2 × 10^5 M^{-1} s^{-1} for $^{\bullet}NO_2$ and Tyr; *k*e, 6.5 × 10^8 M^{-1} s^{-1} for $^{\bullet}OH$ and Tyr; *k*f, 4.5 × 10^7 M^{-1} s^{-1} for $CO_3^{\bullet-}$ and Tyr; *k*g, 3.0 × 10^9 $M^{-1}s^{-1}$. Uric acid protection of Tyr niration by scavenging of $^{\bullet}NO_2$ and $^{\bullet}OH$ (marked in green). Below we represented four putative scenarios in which Pro could avoid nitration of Tyr by the scavenging of different molecules (marked in red). Scenario 1, if Pro reacted with peroxynitrite the formation of radicals compounds that form $^{\bullet}$Tyr would be reduced. Scenario 2, if Pro reacted with $^{\bullet}NO_2$, nitration of $^{\bullet}$Tyr would be reduced. Scenario 3, if Pro scavenged $O_2^{\bullet-}$, formation of peroxynitrite would be reduced. Scenario 4, if Pro scavenged $^{\bullet}NO$, formation of peroxynitrite would be reduced.

Finally, although $O_2^{\bullet-}$ and $^{\bullet}NO$ react very fast, with a rate constant of 4.3 x 10^9 M^{-1} s^{-1} (Fig. 4, *k*a) (Goldstein and Czapski, 1995), the concentration of these molecules in this experiment cannot reach more than the µM range, while Pro concentration is 100 mM (at least 1000-fold grater). So a hypothetical rate constant for the reaction of Pro

and these molecules in the order of 10^6 M^{-1} s^{-1} would compete with the formation of peroxynitrite. Several plant phenols such as delphinidin, epicatechingallate, myricetin, rutin, quercetin, gallic acid and proanthocyanidins have rate constants for reaction with $O_2^{\bullet-}$ greater than 10^6 M^{-1} s^{-1}, i.e. 2.9 x 10^7 M^{-1} s^{-1} for proanthocyanidins (Taubert et al., 2003). A good scavenger of $O_2^{\bullet-}$ or $^{\bullet}NO$, like the molecules mentioned above, would reduce the formation of peroxynitrite and eventually protein nitration (Fig. 4, Scenario 3 and 4 respectively). So as Pro could not prevent protein nitration, we can conclude that Pro does not have the capacity to protect against neither $O_2^{\bullet-}$ nor $^{\bullet}NO$. This partly explains the absence of evidence for rate constants between $O_2^{\bullet-}$ or $^{\bullet}NO$ and Pro at physiological conditions.

In accordance with the results of our *in vitro* assay, *in vivo* experiments were carried out to elucidate the protective mechanisms of Pro against saline stress (NaCl 200 mM), revealing that exogenous Pro (20 mM) did not change $O_2^{\bullet-}$ or $^{\bullet}NO$ levels in tobacco Bright Yellow-2 cells (Banu et al., 2010).

In summary, our work shows that Pro does not prevent protein nitration and only would contribute to reduce $^{\bullet}OH$ toxicity. In a plant cellular context there are many low molecular weight compounds which can also react non-specifically with $^{\bullet}OH$. In this assay low molecular weight compounds of crude extract were removed maximizing the protective role of Pro against $^{\bullet}OH$. In addition, peroxynitrite *in vivo* can react directly with several molecules with relatively high rate constants, much higher than the homolysis rate constant, so that the contribution of the $^{\bullet}OH$ pathway to peroxynitrite toxicity in a cellular context is minimal, and it is considered that most peroxynitrite will react before homolyzing (Alvarez and Radi, 2003). These facts reveal that the cellular context poses a greater challenge for Pro than *in vitro* experiments to prevent protein nitration.

Instead, it is known that Pro contributes to maintaining an adequate redox balance (Sharma et al., 2011). Its biosynthesis generates $NADP^+$, which can be used as final acceptor of electrons in the photosynthetic electron chain (Hare et al., 1998). Also the Pro-Pro cycle, produced in the scavenging of $^{\bullet}OH$ by Pro, contributes to $NADP^+$ regeneration (Signorelli et al., 2014). In this way, increased availability of $NADP^+$ by

Pro accumulation would reduce ROS formation by photosynthetic electron leakage. Lower production of ROS in chloroplasts would result in lower damage of the photosynthetic machinery and therefore in greater tolerance to the stress condition. In contrast, Pro accumulation is known to activate the Pro-P5C cycle which produces ROS by electron leakage in mitochondria (Miller et al., 2009). However, recent evidence suggested that the production of ROS via activation of Pro-P5C cycle may induce antioxidant enzymes giving more tolerance to stress conditions (Khavari-Nejad et al., 2013).

Returning to our question, we consider that it is more likely that Pro accumulation alleviates oxidative damage due to reduction of ROS production in chloroplasts and/or the induction of antioxidant enzymes than because it has antioxidant properties.

Conclusions

Briefly, Pro is not an efficient scavenger of peroxynitrite, $^{\bullet}NO$, $O_2^{\bullet-}$ and $^{\bullet}NO_2$. Further investigation will be required to understand how Pro accumulation can mitigate oxidative damage in stress conditions, either by reducing ROS formation or inducing the expression of antioxidant enzyme genes but not as a general antioxidant.

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CONCLUSIONES

Capítulo 1. Respuestas a sequía de leguminosas

En las leguminosas forrajeras trébol y lotus, la respuesta antioxidante enzimática y la actividad del fotosistema II contribuyen a explicar su diferente tolerancia-sensibilidad a la sequía cuando las temperaturas son altas.

En estreses combinados las respuestas de las leguminosas son variadas, con efectos aditivos, sinérgicos e incluso antagónicos, comparados a cuando se establecen de manera individual. Como la sequía y altas temperaturas por lo general ocurren simultáneamente, es necesario evaluar las respuestas de la planta en esa condición.

En *L. japonicus* se identificaron 2 genes que codifican para P5CS, enzimas que participan de la vía de síntesis de prolina: *Ljp5cs1* inducible en condición de estrés osmótico y responsable de la acumulación de prolina y *Ljp5cs2* constitutivo.

La sequía induce estrés nitro-oxidativo diferencial en *L. japonicus*: mientras que en raíces es mayor el daño nitrosativo, en hojas es mayor el oxidativo.

Capítulo 2. La prolina en la protección frente al daño nitro-oxidativo

El rol antioxidante de la prolina debe ser reconsiderado en la medida que no protege frente al oxígeno singulete, superóxido, óxido nítrico, peroxinitrito ni dióxido de nitrógeno. Estas evidencias permitirán orientar el planteo de nuevas hipótesis sobre el rol de la prolina en plantas.

La protección que ofrece la prolina como antioxidante está dada por la capacidad de captar al $^{\cdot}OH$, lo que se ve favorecido por la concentración que alcanza la prolina en condiciones de estrés.

Propusimos un ciclo, al que denominamos Pro-Pro, que capta 2 $^{\cdot}OH$ y permite la regeneración de $NADP^{+}$ el cual contribuye a mantener la actividad fotosintética y disminuir la generación de ROS por fuga de electrones.

En plantas el GABA se produce por la acción de la glutamato decarboxilasa. Nosotros relacionamos la síntesis y acumulación de GABA con la prolina, a través de otra vía que consiste en la descarboxilación de la prolina por reacción de esta con $^{\cdot}OH$ sobre su grupo amino, seguida de dos reacciones.

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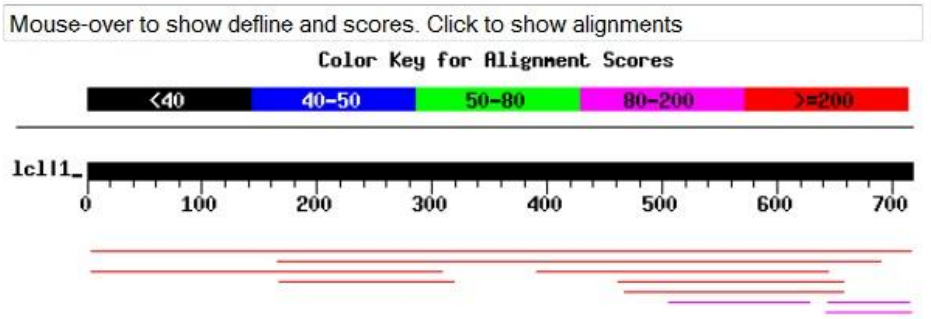
APENDICE

Potenciales secuencias p5cs en *L. japonicus*

Secuencias proteicas de L. japonicus homologas a la P5CS A (717 aa) de *A. thaliana*:

Distribution of 10 Blast Hits on the Query Sequence



Sequences producing significant alignments:	Score (bits)	E Value
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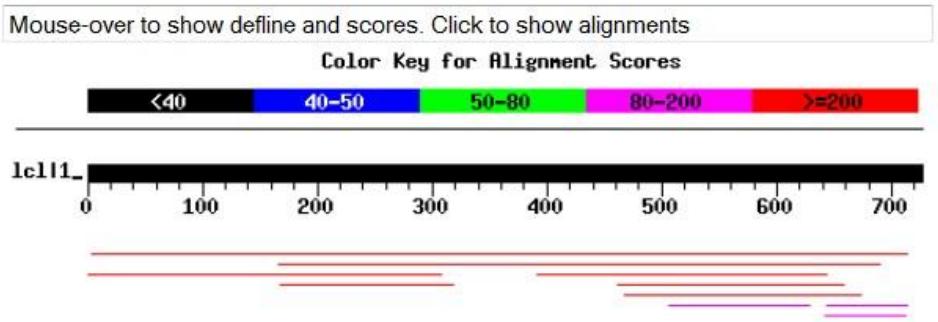
chr2.CM0011.280.r2.m + phase: 0	648	0.0
LjSGA_032463.1 + phase: 0 /pseudo/partial	377	e-104
LjSGA_006172.2 + phase: 2 /partial	354	8e-98
chr2.CM0803.760.r2.m - phase: 2 /partial	216	3e-56
chr4.CM0179.70.r2.a - phase: 0 /partial	204	7e-53
LjSGA_045289.1 + phase: 0 /pseudo/partial	202	5e-52
LjSGA_136569.1 - phase: 1 /pseudo/partial	169	3e-42
LjSGA_053689.1 - phase: 2 /partial	121	1e-27
LjSGA_068114.2 - phase: 1 /partial	117	2e-26

Secuencias proteicas de L. japonicus homologas a la P5CS B (726) de *A. thaliana*:

Distribution of 10 Blast Hits on the Query Sequence



Sequences producing significant alignments:	Score (bits)	E Value
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chr2.CM0011.280.r2.m + phase: 0	666	0.0
LjSGA_032463.1 + phase: 0 /pseudo/partial	388	e-108
LjSGA_006172.2 + phase: 2 /partial	352	4e-97
chr2.CM0803.760.r2.m - phase: 2 /partial	226	2e-59

chr4.CM0179.70.r2.a - phase: 0 /partial	215	4e-56
LjSGA_045289.1 + phase: 0 /pseudo/partial	206	3e-53
LjSGA_136569.1 - phase: 1 /pseudo/partial	179	3e-45
LjSGA_053689.1 - phase: 2 /partial	124	1e-28
LjSGA_068114.2 - phase: 1 /partial	119	3e-27

Secuencia *Ljp5cs 1*:

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